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**GROWTH, INHIBITION AND PATHOGENICITY OF  
MICROORGANISMS IN ENTERAL NUTRIENT SOLUTIONS**

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A thesis submitted in partial fulfilment of the requirements of the Open University for the  
Degree of Doctor of Philosophy

June 1999

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Edinburgh.

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## **DECLARATION**

I declare that this thesis has been composed by myself and that the research reported therein has been conducted by myself.

Edinburgh, 20 June, 1999

**The writing of books is endless and much study is wearisome to the flesh.**

**Ecclesiastes 12:12 The Bible**

## **Acknowledgements**

Firstly, I would like to thank my dedicated supervisors Dr Lorna Fyfe and Dr John Stewart. They have imparted their collected wisdom and have supported me through the traumas of registering and finally completing this thesis. Secondly, I would like to thank Mr Robert Brown, Mr Mike Kerr, Dr Catherine Taylor and Dr Ian Poxton for the practical and technical advice they have given me.

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## **Abstract**

Enteral nutrient solutions (ENS) which are contaminated with microorganisms from exogenous sources or from the microbial flora of the patient's own gastrointestinal tract are associated with bacteraemia, diarrhoea, respiratory infections and septicaemia. These infections seriously undermine some of the advantages of enteral feeding by increasing patient morbidity, mortality and lengthening hospital stay. The strategies which have been employed to prevent contamination of enteral feeding systems and the subsequent incidence of disease have been shown to be ineffective or impractical in a clinical environment. Therefore, this thesis investigated the possibilities of using novel antimicrobial agents (oil of fennel and parabens) to inhibit the growth of microorganisms that commonly contaminate ENS and/or to alter the expression of products which mediate in their pathogenicity. In order to achieve this, the study of growth and the virulence determinants of microorganisms in enteral feeding solutions was necessary. Gram-negative bacteria are the most important group of microorganisms that contaminate ENS and therefore this group of bacteria and lipopolysaccharide, an important Gram-negative bacterial virulence determinant, were particularly singled out for study.

The results showed that the growth of all test strains of microorganisms was similar in milk-based enteral nutrient solutions and laboratory media, while only *Candida albicans* and *Klebsiella aerogenes* grew in the "fruit-based" ENS. None of microbial strains were inhibited in milk-based ENS by the concentrations of oil of fennel or parabens used singly. However, oil of fennel and parabens used in combination had synergistic antimicrobial activity and inhibited the growth of all microbial strains.

Growth in milk-based enteral nutrient solutions was found to alter the phenotypic expression and biological activity of lipopolysaccharide. Results showed differences in the O-polysaccharide portion of the lipopolysaccharide of bacteria cultured in milk-based enteral nutrient solutions compared to those cultured in laboratory media. Bacteria cultured in milk-based enteral feeds had increased serum resistance and also induced a

significantly greater release of nitric oxide from a human monocyte cell line than bacteria cultured in laboratory media. The concentrations of oil of fennel and methyl parabens used in experiments did not alter the expression of O-polysaccharide.

Enteral nutrient solutions can therefore, not only support the growth of microorganisms but alter virulence determinants. This could have important consequences for immunocompromised patients who receive ENS.

## **Publications arising from this research**

- Hodgson I, Stewart J, and Fyfe L, (1995). Synergistic antimicrobial properties of oil of fennel and parabens: the potential for food preservation. Poster presentation at "Disinfection, Sterilization and Preservation: Problems and Practices" Society of Applied Microbiology Conference, University of Surrey, Guildford, UK.
- Hopper H, Hodgson I, Stewart J, and Fyfe L, (1995). Inhibition of *Candida albicans* by food flavourings in a milk-based enteral feed. Poster presentation at the Royal Society of Chemistry, London.
- Hodgson I, Stewart J, Fyfe L, (1997). Phenotypic changes in *Pseudomonas aeruginosa* Lipopolysaccharide grown in milk-based enteral feeds. The Proceedings of the Nutrition Society 56:238A. Poster presentation at the Annual Conference of the British Association of Parenteral and Enteral Nutrition (BAPEN). Received 1st Prize for poster presentations.
- Hodgson I, Stewart J and Fyfe L (1998). Inhibition of bacteria and yeast by oil of fennel and paraben: development of synergistic antimicrobial combinations. J. Essential Oil Research. 10: 293-297.
- Hodgson I, Stewart J and Fyfe L (1999). Phenotypic changes in the lipopolysaccharide of *Pseudomonas aeruginosa* and *Escherichia coli* grown in milk-based enteral nutrient solutions. Nutrition. 15: 11-17.
- Hodgson I, Stewart J and Fyfe L (1999). Synergistic combinations of oil of fennel and methyl parabens: the potential for preservation of milk-based enteral nutrient solutions. *In preparation*.
- Hodgson I, Stewart J and Fyfe L (1999). Changes in the biological activity of virulence factors from *Escherichia coli* grown in milk-based enteral nutrient solutions. *In preparation*.

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# CHAPTER 1

## GENERAL INTRODUCTION

### 1.1 NUTRITIONAL SUPPORT OF PATIENTS

The need for nutritional support may arise as a result of surgical procedures, trauma, burns or because the patients are unable to feed themselves adequately. Patients included in the latter group are neonates, the elderly and the neurologically impaired. There is also a significant and growing role for nutritional support during the treatment of underlying diseases and conditions such as cancer, inflammatory bowel diseases, pancreatic disorders, liver failure, renal disease, diabetes mellitus, Acquired Immune Deficiency Syndrome (AIDS) and respiratory disease (McCamish *et al*, 1997). Nutritional support in these contexts has been shown to be beneficial in reducing mortality, morbidity and hospital stay length (Minard and Kudsk, 1994; Giner *et al*, 1996). Nutritional support of patients may be achieved by using Total Parenteral Nutrition (TPN) or enteral nutrition. TPN is the intravenous administration of nutrient solutions into the patient, whereas, enteral nutrition is the introduction of elemental or partially hydrolysed diets into the gastro-intestinal tract (GI-tract) of the patient.

For a number of reasons enteral feeding is now considered preferable to TPN in the majority of patients (Suchner *et al*, 1996). Firstly, it is superior in preserving the integrity and function of the gut by maintaining lower intestinal mass (Levine *et al*, 1974) and decreasing permeability (Hadfield *et al*, 1995). These are both essential for prevention of bacterial translocation from the gut (Deitch *et al*, 1995), reducing the risk of post-operative disturbance of the small bowel and improving visceral protein synthesis and carbohydrate homeostasis (Suchner *et al*, 1996). Secondly, work in animal models indicates that immune responses to bacterial challenge from the gut micro-flora are better in the enterally fed. Lin and co-workers (1996) studied the response of enterally fed rats to peritoneal bacterial challenge. They reported increased numbers of peritoneal

exudative cells (PEC), increased cytokine production by PECs and better regulation of Tumour Necrosis Factor (TNF) at the site of the infection. In addition, immune cells in remote organs were mobilised to the peritoneal cavity more effectively, systemic TNF production was diminished and interferon (INF- $\gamma$ ) production was enhanced. In their study of 49 patients, Suchner *et al*, (1996) speculated that increased platelet counts of enterally fed patients compared to TPN patients indicated a lower inflammatory response. Finally, it is generally accepted that enteral feeding is associated with fewer and less severe complications (Heberer and Harder, 1986) and lower costs (Twomney and Patching, 1985).

Although there are many advantages to enteral feeding compared to TPN, enteral feeding is not entirely without risks. One of the most important risks of enteral feeding arises when the enteral nutrient solution or feeding system becomes contaminated. This risk will be discussed further in the following sections and chapters.

## **1.2 ENTERAL FEEDING SYSTEMS AND PRACTICE**

The first documented case of enteral nutrition is that of ancient papyri describing nutrient enemas administered by Egyptian doctors in 1500 BC. Rectal feeding continued as the principal method of administering enteral feedings well into the 20th century. The enteral foods administered in this manner were as diverse as milk, grain, meat broths, whey, whisky, brandy and finely ground pig or cow pancreas (McCamish *et al*, 1997 citing Bliss, 1882; and Brown-Sequard, 1887).

The modern concept of enteral feeding only began to develop at the end of 18th century when John Hunter developed a method for administering foods oro-gastrically using an eel-skin covered whale bone. Even so, enteral feeding remained a rudimentary affair for the next century, helped only by the invention of the stomach pump at the end of the 18th century and the introduction of rubber and gum-elastic catheters (McCamish *et al*, 1997 citing Clouston, 1872). In 1910, Max Einhorn made a significant breakthrough in the field of enteral nutrition. By using a weighted rubber tube to feed patients a milk, egg, and lactose preparation directly into the duodenum, he negated the need to feed patients rectally when gastric feeding was not possible (McCamish *et al*, 1997 citing Einhorn, 1910).

Tube feeding technology has improved considerably since these early times. Enteral feeding systems used by the clinicians of today comprise a pump, feeding set and administration containers. Enteral foods or enteral nutrition solutions can either be decanted into reservoirs from bottles, cans or tetra-brik cartons, or used in a "ready-to-hang" format. Silicon and polyurethane fine-bore tubes which are more easily tolerated by the patient and more flexible when exposed to the conditions of the GI-tract have replaced rubber and PVC tubes. Many naso-enteric tubes have features such as multiple eyelets to enhance feeding outflow from the distal end of the tube, Y-ports to allow the administration of medication and irrigation of tubes and radio-opaque materials to enable radio-placement of tubes (McCamish *et al*, 1997). Techniques such as Percutaneous

Endoscopic Gastrostomy (PEG) have revolutionised long-term feeding and the enteral support of patients with a high risk of aspiration (Ponsky *et al*, 1985; and Gauderer *et al*, 1998).

### **1.2.1 Routes used in enteral feeding**

The route selected for the administration of enteral nutrient solutions depends upon the integrity of the gastrointestinal tract, the anticipated duration of feeding and the potential for aspiration in the patient (American Society of Parenteral and Enteral Nutrition: ASPEN, Directors Report, 1993). There are two means of enteral feeding: sip and tube feeding.

#### **1.2.1.1 Sip feeding**

Sip feeding is the consumption of enteral nutrient solutions from tetra-brik packages or cans using a straw. Sip feeds are utilised when patients are unable to tolerate solid foods or require increased energy intake to counteract weight loss. Sip fed patients should have good gastro-intestinal integrity and a low risk of aspiration. Sip feeds are commonly prescribed for nutritional support of the elderly, those with cancer and those with AIDS.

#### **1.2.1.2 Tube Feeding**

The majority of tube feeding systems consist of a reservoir of enteral nutrient solutions, a tube connecting the reservoir to the feed catheter, an infusion pump to maintain a constant flow of enteral nutrient solution to the patient and a feed catheter which is inserted into the patient's GI tract. In many cases, tubes are inserted via the nasopharynx into the stomach, duodenum or jejunum. Feeding via the nasopharynx is suitable for patients where the risk of aspiration is low and is the preferred method of tube feeding for patients who will resume normal oral feeding (ASPEN Directors, 1993). Alternatively, if the patient is expected to receive long term feeding or has a high risk of aspiration, surgical insertion of the tube into the GI-tract is required (ASPEN Directors, 1993). This is achieved by making a percutaneous port into the stomach, duodenum or

jejunum using either invasive surgical techniques (gastrostomy, duodenostomy, jejunostomy) or endoscopic techniques (PEG, PED or PEJ).

### **1.2.2 Enteral tube-feeding systems**

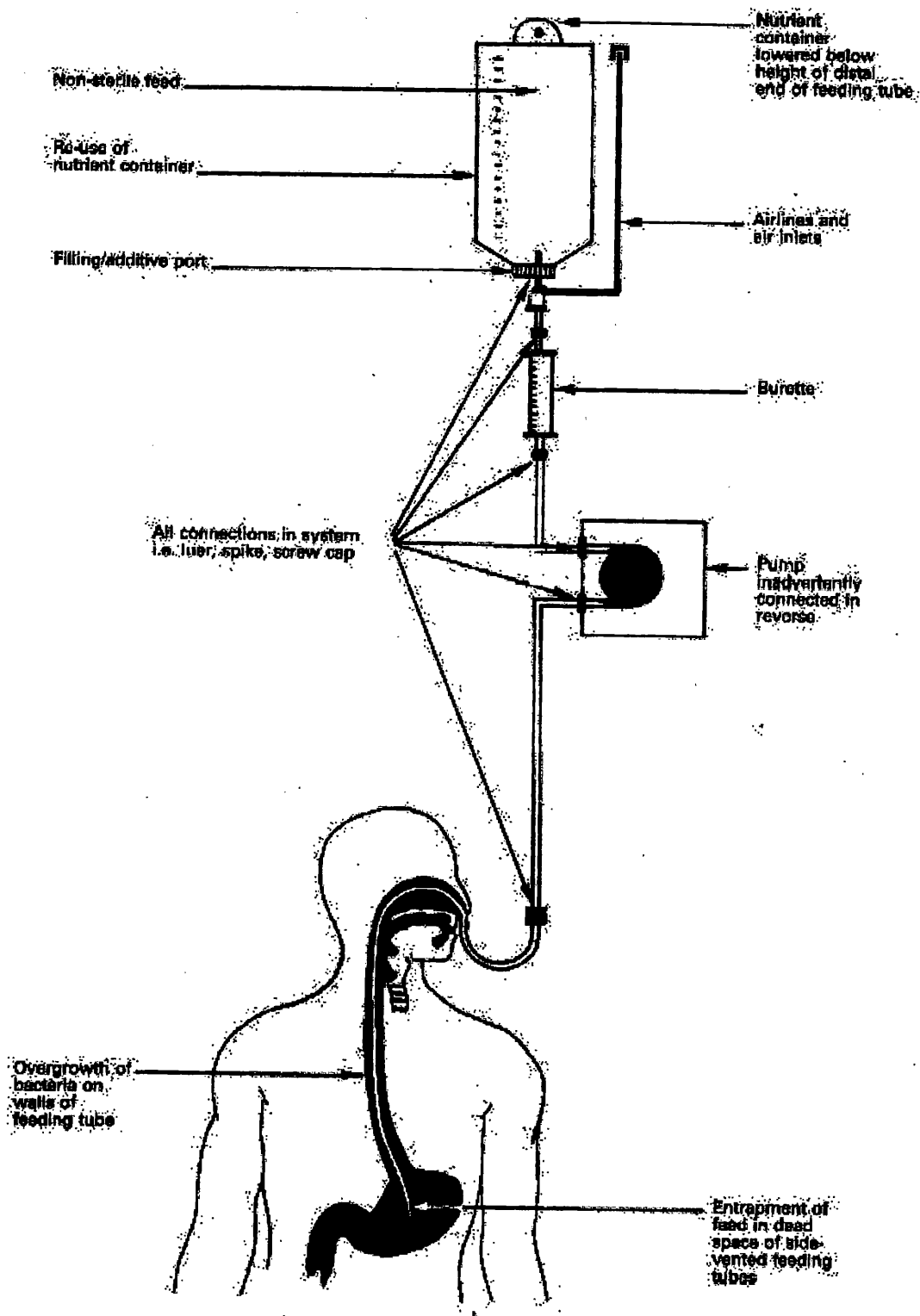
There is a vast array of enteral feeding equipment available to the clinician, marketed on the basis of performance, flexibility, ease of use and, more recently, microbiological safety. Enteral feeding systems can be categorised into "open" feeding systems and "closed" feeding systems. Open feeding systems have refillable reservoirs (flexible bags or rigid containers) and can be used with ready-to-use enteral nutrient solutions or with formulas prepared from powdered ingredients in the hospital or home (Figure 1.1). These systems are open to the air and require considerable amounts of manipulation to set up and administer. In contrast, "closed" feeding systems have completely enclosed reservoirs which usually consists of a bottle or tetra brik pack that can be connected or "spiked" with the giving set. Some manufacturers produce "closed" systems with an integral feeding set (Figure 1.2). "Closed" systems are therefore, easier to set up and time-saving but also more expensive.

### **1.2.3 Products used in enteral feeding systems: Enteral Nutrient Solutions**

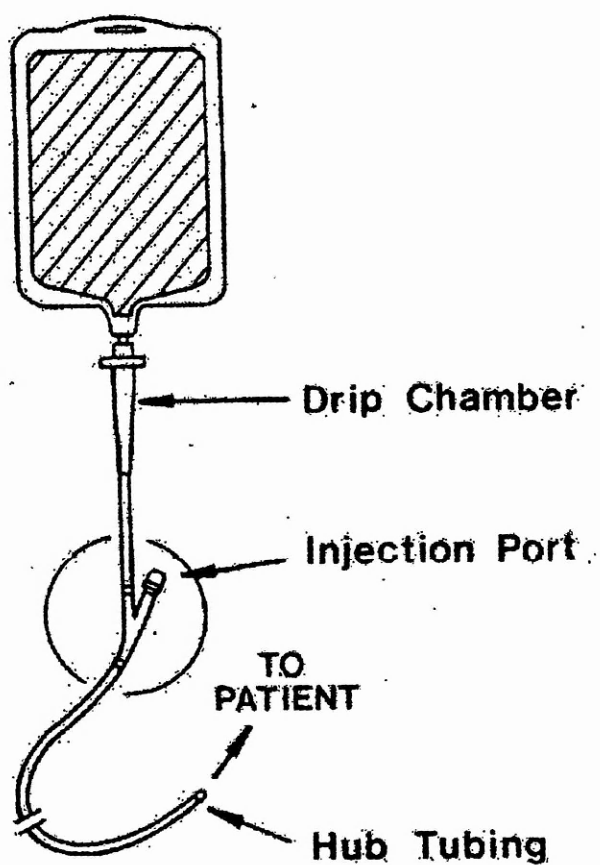
Enteral nutrient solutions are of diverse composition, reflecting the range of patients who are fed or treated by them. They may be reconstituted in the hospital or home from powders (mixed-by-hand enteral nutrient solutions) or produced aseptically by a manufacturer and packaged in cans, bottles, tetra-briks or retortable pouches with integral giving sets (ready-to-use enteral nutrient solutions).

Enteral nutrient solutions should be considered as foods that are designed to meet the specific nutritional needs of the patient while supporting the integrity of the gut and the metabolic function of organs elsewhere in the body (ASPEN Directors, 1993). As such, enteral nutrient solutions may be elemental in nature consisting of simple sugars, amino acids, simple peptides and fatty acids, or more complex. More complex enteral nutrient





**Figure 1.1:** An “open” enteral feeding system showing access points for microbial contaminants. Adapted from Anderton *et al* (1986).



**Figure 1.2:** A “closed” enteral feeding system (Entripak, Biosearch Medical Products Incorporated, Somerville, New Jersey, USA). Adapted from Crocker *et al* (1986).

solutions consist of whole or partially hydrolysed proteins originating from milk or soya and it is these types of feeds which are the subject of this thesis.

Complex enteral nutrient solutions may be subdivided into milk-based and "fruit"-based enteral nutrient solutions. Milk-based enteral nutrient solutions are generally bland emulsified mixtures of vegetable oils (canola, sunflower and coconut oils), hydrolysed polysaccharides (maltodextrins) and whole caseinates or soya proteins. They also contain a wide range of micronutrients, are neutral in pH and are used both as sip feeds and tube feeds. In contrast, "fruit"-based enteral nutrient solutions are highly flavoured mixtures of hydrolysed proteins (soya, whey, casein or mixtures of these), hydrolysed polysaccharides (maltodextrins), disaccharides (maltose and sucrose) and citric acid. They also contain a wide variety micronutrients but are of low pH (typically pH 4). "Fruit"-based products are only used as sip feeds.

### **1.3 MICROBIAL CONTAMINATION OF ENTERAL FEEDING SYSTEMS AND ENTERAL NUTRIENT SOLUTIONS**

Enteral feeding systems can become contaminated with a wide range of Gram-positive and Gram-negative microorganisms. These are environmental organisms or microbes that form part of the normal human commensal floras of the gut and skin (Anderton, 1993). A summary of these organisms is shown in Table 1.1. Microorganisms originating from contaminated ingredients or equipment and from the environment or administering personnel are termed "exogenous contamination". Conversely, microorganisms which originate from the patient's own gastro-intestinal tract and enter the feeding system are termed "endogenous" contamination (van Alsenoy *et al*, 1985; Payne-James *et al*, 1992; Beattie *et al*, 1996). Microorganisms may contaminate the feeding systems by forming adherent crusts in feeding tubes and catheters, thereby contaminating the enteral nutrient solution flowing in the feeding system. Yeasts, particularly *Candida albicans*, are viewed as the predominant type of microorganisms which colonize feed catheters and tubing (Anderton, 1986b).

The great variation in products, feeding methods and practice employed in enteral feeding means that it is not surprising that the reports of the incidence of contamination range from 0-90% (Weenk *et al*, 1995; Freedland *et al*, 1989).

#### **1.3.1 Sources and mechanisms of exogenous contamination**

The risk of contamination of enteral feeding systems during preparation and administration of the enteral feed is multifactorial (Grunow *et al*, 1989). Increased risk of contamination is associated with a number of factors and procedures which have been extensively reported in the literature.

**Table 1.1:** Microbial species isolated from a variety of enteral nutrient solutions

<b>MICROORGANISM</b>	<b>REFERENCE.</b>
<i>Acinetobacter baumannii</i>	(14) (16)
<i>Acinetobacter</i> spp.	(9) (21) (22)
<i>Aeromonas</i> spp.	(4) (21)
<i>Bacillus cereus</i>	(4)
<i>Bacillus</i> spp.	(4) (5) (8) (14) (21) (22)
<i>Candida albicans</i>	(3)
<i>Candida</i> spp.	(3)
<i>Clostridium difficile</i>	(7) (12)
<i>Corynebacterium</i> spp.	(9)
<i>Citrobacter</i> spp.	(1)
<i>Enterobacter cloacae</i>	(1) (2) (6) (8) (13) (14) (16)
<i>Enterobacter</i> spp.	(1) (4) (5) (8) (10)
<i>Enterococcus</i> spp.	(2) (9)
<i>Escherichia coli</i>	(1) (4) (8) (10)
<i>Klebsiella aerogenes</i>	(11)
<i>Klebsiella pneumoniae</i>	(1) (5) (9) (14) (22)
<i>Klebsiella</i> spp.	(8)
<i>Lactobacillus</i> spp.	(20)
<i>Leuconostoc</i> spp.	(20)
<i>Moraxella</i> spp.	(4)
<i>Proteus</i> spp.	(1) (9)
<i>Pseudomonas aeruginosa</i>	(8) (21) (22)
<i>Pseudomonas</i> spp.	(1) (4) (9) (14) (21) (22)
<i>Providencia</i> spp.	(4)
<i>Salmonella enteritidis</i>	(15)
<i>Serratia marcescens</i>	(2) (8) (9) (14)
<i>Staphylococcus aureus</i>	(3) (4) (5) (8)
<i>Staphylococcus epidermidis</i>	(1) (8) (21) (22)
<i>Staphylococcus</i> spp.	(1) (2) (9) (21)
<i>Streptococcus viridans</i>	(22)
<i>Streptococcus faecalis</i>	(22)
<i>Streptococcus</i> spp.	(2) (4) (5) (8) (9) (13)
<i>Xanthomonas</i> spp.	(4)

(1) Anderson *et al* (1984); (2) van Alsenoy *et al* (1985); (3) Anderton (1984); (4) Pottecher *et al* (1979); (5) Anderton (1993); (6) Casewell *et al* (1981); (7) Edes *et al* (1990); Van Enk *et al* (1986); (9) Freedland *et al* (1989); (10) de Vries *et al* (1982); (11) Kiddy *et al* (1987); (12) Kohn, (1991); (13) Marcuard *et al* (1993); (14) Thurn *et al* (1990); (15) Gill and Gill (1981); (16) Levy *et al* (1989); (17) Schreiner *et al* (1979); (18) Baldwin *et al* (1984); (19) Gutman *et al* (1976); (20) Carapetis *et al* (1994); (21) Hostetler *et al* (1982); (22) Chan *et al* (1994).

### 1.3.1.1 Contaminated Ingredients

Mixed-by-hand products are powders reconstituted with water. This type of enteral nutrient solution is rarely used for adult patients but is commonly administered to neonates (Infant Milk Formulas). Infant Milk Formulas are specialised milk products utilising whey proteins and have been shown to be contaminated with *Bacillus* spp. at concentrations of up to 10,000 cfu per g (Anderton (1993) citing MacBurney *et al*, 1990; Bastow *et al*, 1982; Anderton, 1985; Rowan *et al*, 1997). In the study of Rowan and co-workers (1997) the pathogenic *B. cereus*, which produces an enterotoxin, was detected in 2 out of the 24 feeds tested in a Glasgow hospital. Similarly, in a survey of Infant Milk Formulae collected from 28 countries, Muytjens and co-workers (1988) found that 52.5% were contaminated with enterobacteriaceae. However, contamination does not only arise from the powder itself but also from the water used to rehydrate the formula. Tap water often contains high numbers of non-pathogenic organisms. However, opportunistic pathogens such as *K. pneumoniae* and *P. aeruginosa* may also be present in lower numbers (Ptak *et al*, 1973; Olson and Hanami, 1980). Even bottled drinking water commonly contains *Pseudomonas* spp., *Flavobacterium* spp. and *Alcaligenes* spp. and is thus considered an unsuitable water source (Anderton, 1993). Recommendations for microbiological control in enteral feeding direct that sterile water should be used on all occasions (Anderton *et al*, 1986).

### 1.3.1.2 Feed preparation equipment

Enteral nutrient solutions that are mixed-by-hand may also become contaminated because the equipment used to prepare them is also contaminated. In particular, the use of electric blenders has been linked to the contamination of enteral formulas (Kiddy *et al*, 1987; Anderton and Aidoo, 1991a). In one case, Casewell and co-workers (1981) described a patient with nosocomial septicaemia caused by *Ent. cloacae* originating from a blender. Blenders may provide a reservoir for microorganisms because the cleaning and disinfection procedures commonly employed in hospitals are inefficient at removing organisms (Anderton and Aidoo, 1991a). This research has emphasised, so far as

microbial safety is concerned, the superiority of ready-to-use formulas as opposed to those that are mixed-by-hand.

#### 1.3.1.3 Decanting

Enteral nutrient solutions (mixed-by-hand or ready-to-use) used in "open" feeding systems must be decanted into the feed reservoir. This procedure has been shown to carry a particularly high risk so far as microbial contamination of enteral feeds is concerned (Navajas *et al*, 1992). Enteral nutrient solutions may become contaminated during decanting as a result of organisms being present on the surface of packaging (ready-to-use) or on the outside of preparation equipment (Anderton and Aidoo, 1990). Anderton and Aidoo (1990) suggest that this type of contamination may be prevented by wiping the surface of any packaging with alcohol wipes and using sensible precautions when preparing feeds such as washing hands and the use of gloves.

#### 1.3.1.4 Administering personnel and manipulation of the feeding system

The hands of nurses and other personnel, who are involved in the administering of enteral feeds to patients, are recognised as one of the most important sources of microorganisms which contaminate enteral nutrient solutions. Casewell and Philips (1977), Sanderson and Weissler (1992) and Scott and Bloomfield (1990) have all shown that a wide range of potentially pathogenic microorganisms present on nurses' hands can be transferred to various surfaces without significant loss of viability. Therefore, it is no surprise that Schroeder *et al* (1983) and Thurn *et al*, (1990) isolated the same microorganisms in contaminated enteral nutrient solutions, as those present on the hands of nurses who were manipulating the feeding systems. In particular, Thurn *et al*, (1990) identified the opportunistic pathogens *A. baumannii*, *Enterobacter* spp., *Klebsiella* spp., *P. aeruginosa* and *S. marcescens* as being present both on the hands of nurses and in the enteral feeds they were administering.

Both the studies of Crocker *et al*, (1986) and Grunow *et al*, (1989) considered the number of manipulations of an enteral feeding system received during the assembly and administration of enteral formulas to have a positive correlation with incidence of contamination. This view is supported by the work of Anderton and Aidoo (1988) who experimentally contaminated hands with *K. aerogenes* or *E. coli* and found that normal manipulation of an enteral feeding system resulted in  $10^3$  cfu ml<sup>-1</sup> concentrations of these microorganisms in the enteral nutrient solution after 4 hours of perfusion. These authors concluded that airway tubes, flutter valves, spikes and caps served as potential routes for microbial contaminants to enter the feeding systems during manipulation. Similarly, Chan and co-workers (1994) found that microorganisms could enter Y-ports in a pre-filled ready-to-use feeding system. However, contrary to the reports of Chan and co-workers (1994), Donius, (1993) recommends the inclusion of Y-ports to reduce the need for manipulation and thus, the incidence of contamination in enteral nutrient solutions. Both of these reports are indicative of the diversity of opinion about whether design modifications by enteral feed system manufacturers have reduced the importance of exogenous contamination.

### **1.3.2 Source and mechanisms of endogenous contamination**

A study by Schroeder and co-workers (1983) was the first to propose that contamination of enteral feeds could occur as a result of retrograde movement of microorganisms from the patient's own gastro-intestinal tract into the feed catheter, tubing and reservoir. However, attempts by these authors to simulate the retrograde movement of organisms into feed catheters in the laboratory failed, and they concluded that such a mechanism was unlikely to occur. In contrast to this report, more recent laboratory and clinical studies have shown that retrograde movement of organisms not only happens but is perhaps the most important mechanism of contamination in modern enteral feeding systems (van Alsenoy *et al*, 1985; Payne-James *et al*, 1992; Beattie *et al*, 1996). Payne-James and co-workers (1992) found that microorganisms originating from the gut could ascend into the feed reservoir within the normal hang-time (48 hours) of the product but



that this could be prevented by the inclusion of a drip-chamber in the feed lines. However, they also noted that the use of ball-valves to prevent reflux of feed from the patient back into the feeding system counteracted the beneficial effects of the drip chamber.

This type of contamination, as exemplified by a number of studies, is of particular concern because it may lead to giving sets and feed catheters becoming colonised with microorganisms (Anderton, 1984; Anderton, 1986b; Anderton and Nwoguh 1991b; and Marcuard *et al*, 1993). *Candida* spp. were the most important microbial species isolated from tubes in these studies. Although only a minority of these reports speculate about the origins of the microorganisms growing in the tubes, it is very likely that colonization occurred by retrograde spread of microorganisms from the gut of the patients concerned. The retrograde spread microorganisms from the gut and colonization of enteral feeding tubes and catheters should be researched further. Little is known about how this type of contamination occurs, how it may be eliminated, or the implications of such contamination. Organisms present on the inner lumen of enteral feeding tubes have been shown to be resistant to attempts to clean or kill them (Anderton and Nwoguh 1991b) and may "seed" enteral nutrient solution as it flows past. These microbial crusts have been shown to penetrate the silicone material of the catheter causing the tube to lose flexibility and in some cases break (Marcuard *et al*, 1993). In addition, organisms present on the outside surface of nasogastric catheters may be able to cause pulmonary infections (Anderton, 1993; Payne-James *et al*, (1992).

### **1.3.3 Other factors which facilitate contamination**

#### **1.3.3.1 The use of "open" systems versus "closed" systems**

"Open" enteral feeding systems are more likely to become contaminated with microorganisms because they may be used in conjunction with contaminated mixed-by-hand type formulas; feeds must be decanted into the feed reservoir; and they require

more manipulation during administration of the feed (Anderton, 1993; Donius, 1993). This is discussed further in Section 1.5.1.2.

### 1.3.3 2 The feeding environment (Home feeding versus hospital feeding)

Home enteral feeding has been accepted as an efficient and relatively inexpensive way for certain patient groups to receive nutritional support (Newmark *et al*, (1981). In particular, overnight nasogastric feeding at home negates the need for prolonged hospital stays and improves voluntary oral feeding by day (Allison, 1986). Consequently, enteral feeding at home is now common practice. However, the risk of contamination is considered to be greater in the home because of the inexperience of personnel preparing and administering the enteral nutrient solutions and because of poor hygiene, (Anderton, 1990). Anderton and co-workers (1993) compared the levels of contamination in Ensure (Abbott Laboratories Ltd, Queenborough, Kent, UK) supplemented with Maxijul (Scientific Hospital Supplies Ltd, Liverpool, UK) prepared for nasogastric feeding using an "open" feeding system, at home and in the hospital. Their findings demonstrated a higher incidence of microbial contamination in feeds prepared in the home (82% n=22) compared to feeds prepared in the hospital (30%, n=73). In addition, the levels of contamination in the feeds prepared at home were higher ( $10^1$ - $10^6$  cfu ml<sup>-1</sup>) compared to those prepared in the hospital (<10 cfu ml<sup>-1</sup>). However, the organisms observed in both groups were similar, including *Staphylococcus* spp., *Bacillus* spp., *Streptococcus viridans*, *Streptococcus faecalis*, *Klebsiella* spp., *Enterobacter* spp. and *Enterobacter cloacae*. The similarities in microbial flora indicate that the higher incidence and levels in home-prepared feeds is as a result of poor aseptic technique and administration technique rather than unhygienic conditions in the home environment. Studies by Patchell *et al* (1994) and Anderton (1990) conclude that adequate training of administering personnel in good aseptic technique and the use of ready-to-use enteral feeds which are easier to administer would be beneficial in reducing the incidence of contamination in enteral feeds at home.

## **1.4 THE EFFECTS OF ADMINISTERING MICROBIALY CONTAMINATED ENTERAL FEEDS TO PATIENTS**

Microbial contamination in enteral nutrient solutions is important because it is associated with infectious complications which increase patient morbidity and in some cases mortality (Navajas *et al*, 1992; Anderton, 1993). These complications affect the quality of life and increase the cost of enteral feeding, counteracting many of the benefits of enteral nutrition (Mickschl *et al*, 1990).

### **1.4.1. Infections of the gastrointestinal tract: Diarrhoea**

There is evidence to show that once patients have received enteral formula contaminated with microorganisms, their gastrointestinal tracts may become colonized by those organisms (Gutman *et al*, 1976; van Enk and Furtado, 1986). In a clinical study investigating contaminated enteral feedings as a source of nosocomial infections, Thurn and co-workers (1990) found that 62.4% of patients receiving mixed by hand formulas were colonized with organisms present in the feed, while 21.4% of patients receiving ready-to-use formulas were colonized. Colonization of the gut by such microorganisms, especially if they are present in high numbers, may result in a disruption to the balance of the gastro-intestinal micro-flora leading to the establishment of pathogens and an increased risk of diarrhoea.

Enteral feeding is considered a more important cause of diarrhoea in tube-fed patients than *Clostridium difficile* infections caused by prolonged use of broad-spectrum antibiotics (Edes *et al*, 1990; Caines *et al*, 1997). However, Edes and co-workers (1990) found that medications given concomitantly with the enteral formula were more often the cause of diarrhoea than microbial contamination of feeds because they contained high concentrations of sorbitol. The incidence of enteral feeding-related diarrhoea is reported to be as high as 63% (DiSario *et al*, 1990) and as low as 2.3% (Cataldi-Betcher *et al*, 1983). This large discrepancy in accounts of prevalence rates is because of differences in the methods and the definition of diarrhoea, used by the studies in this area (Heimburger,

1990; Bowling and Silk, 1997). Heimbürger (1990) criticises many of these studies for lack of statistical thoroughness, not going beyond statistical associations to establish causal links and often making associations which lack biological plausibility.

The characteristics of the enteral nutrient solutions (Osmolarity and fibre content), intestinal intolerance of the feed resulting in hypoalbuminemia and bacterial contamination of the formula have been proposed as possible causes for enteral formula-related diarrhoea (Kandil *et al*, 1993; Burns and Jaireth, 1994). However, all of these factors have been contra-indicated by other studies. For example, Anderson *et al* (1984) and Navajas *et al* (1992) found a significant correlation between formula contamination and the incidence of diarrhoea, while Freedland *et al* (1989) and Mickschl *et al* (1990) found a poor correlation. Heimbürger (1990) suggests that because bacterial dysentery is seldom found in cases of enteral feeding-related diarrhoea, bacterial contamination is probably not an important factor. However, Kohn (1991) expresses the opinion that, although bacterial contamination may not be responsible for the majority of cases of tube-feeding-related diarrhoea, its importance should not be underestimated. It is clear that before conclusions can be drawn, further research is required to establish causal rather than statistical links between these factors and diarrhoea in the enterally fed. However, the ethical difficulties of such research are obvious.

#### **1.4.2 Systemic Infections**

Colonization of the GI-tract by microorganisms from contaminated feeds may also result in the systemic spread of these organisms and their products throughout the body. Van Enk and Furtado (1986) found that mice fed with enteral nutrient solution contaminated with Group B Streptococcus were rapidly colonized, with a large proportion of the mice going on to develop sepsis and die. Similarly, there are a number of reports of patients developing bacteraemias and septicaemias caused by contaminating microorganisms present in the enteral formulas given to them (Casewell, 1979; Baldwin *et al*, 1984; Kiddy *et al*, 1987; Freedland *et al*, 1989; Levy *et al*, 1989; Carapetis *et al*, 1994).

Organisms particularly associated with this type of infections are *Ent. cloacae* and *K. pneumoniae*. However, a case of particular concern was reported by Carapetis *et al* (1994) who found that a patient became infected with a vancomycin-resistant *Lactobacillus* spp. as a result of being fed a contaminated feed. This is because antibiotic resistance not only makes any subsequent infections difficult to treat but also may facilitate genetic transference of resistance to the microbial flora of the patient's GI-tract.

#### **1.4.3 Respiratory Tract Infections**

It has also been suggested that colonization of the GI-tract with organisms from contaminated feeds may also lead to the development of respiratory tract infections (Thurn *et al*, 1990). These authors found that two out of 24 patients developed pneumonia as a result of receiving enteral feeds contaminated with *A. baumannii*. It is thought that nasogastric enteral feeding promotes the colonisation of the upper respiratory tract and subsequent pneumonia by facilitating colonization of the stomach (Torres *et al*, 1996). This theory is supported by the work of Bonten and co-workers (1994) who found that enteral feeds decreased gastric pH, predisposing the stomach to colonization by gut microbial flora. In addition, Payne-James *et al* (1992) and Beattie *et al*, (1996) suggest that the external surface of a nasogastric catheter may provide a conduit for microorganisms to colonize the upper respiratory tract and cause infection.

## **1.5 EXISTING METHODS WHICH PREVENT MICROBIAL CONTAMINATION OF, OR MICROBIAL GROWTH IN, ENTERAL NUTRIENT SOLUTIONS AND FEEDING SYSTEMS**

In response to reports of nosocomial infections developed as a result of administering contaminated enteral nutrient solutions to patients, strategies have been developed over the last decade to prevent the entry of microorganisms into the feeding system and to inhibit their growth once present.

### **1.5.1 Exclusion of microorganisms from enteral nutrient solutions**

Attempts have been made to exclude microorganisms from enteral feeding systems. These have included microbiological standards and the use of "closed" enteral feeding systems.

#### **1.5.1.1 Microbiological standards**

Recommended microbiological standards have been prepared for ingredients used in non-sterile enteral feeds and for enteral feeds in the nutrient container prior to administration (Table 1.2: Anderton *et al*, 1986). However, it is unclear whether these standards are useful in practice because they require a monitoring program and the maintenance of "best practice" when preparing feeding systems. Anecdotal evidence suggests that despite extensive guidelines which aim to ensure the sterile preparation and administration of enteral products, clinical practice often falls short of these ideals due to the pressures of time and resources.

In addition, these standards do not seem to be widely accepted in the literature. Although it is generally agreed that the presence of any microorganisms (especially pathogenic species) is undesirable, opinions differ as to what concentrations of opportunistic pathogens constitute a threat to enterally fed patients. For example, a number of authors consider concentrations of opportunistic pathogens below  $10^4$  cfu ml<sup>-1</sup> as acceptable (Donius, 1993; Wagner *et al*, 1994), while Anderton *et al* (1986) conclude that the

**Table 1.2:** Recommended microbiological limits for enteral feeds. (Taken from Anderton *et al*, 1986)

Aerobic plate count cfu ml <sup>-1</sup>					
Raw material	Accept	Marginal	Reject	Organisms not permitted at any level	Maximum hang times
Water	<10 <sup>1</sup>	-	≥10 <sup>1</sup>	<i>E. coli</i> <i>Pseudomonas</i> spp.	-
Other liquids	<10 <sup>1</sup>	-	≥10 <sup>1</sup>	<i>E. coli</i> <i>Salmonella</i> spp. <i>Clostridium</i> spp. <i>S. aureus</i> <i>B. cereus</i> <i>Klebsiella</i> spp. <i>Pseudomonas</i> spp.	-
Solids eg powders	<10 <sup>1</sup>	10 <sup>1</sup> -10 <sup>2</sup>	≥10 <sup>2</sup>	<i>E. coli</i> <i>Salmonella</i> spp. <i>Clostridium</i> spp. <i>S. aureus</i> <i>B. cereus</i> <i>Klebsiella</i> spp. <i>Pseudomonas</i> spp.	-
Aerobic plate count cfu ml <sup>-1</sup>					
Product prior to use.	Accept	Marginal	Reject	Organisms not permitted at any level	Maximum hang time.
Sterile feeds	0	-	>0	Any	24 hours
Non-sterile feeds	<10 <sup>1</sup>	10 <sup>1</sup> -10 <sup>2</sup>	≥10 <sup>2</sup>	<i>E. coli</i> <i>Salmonella</i> spp. <i>Clostridium</i> spp. <i>S. aureus</i> <i>B. cereus</i> <i>Klebsiella</i> spp. <i>Pseudomonas</i> spp.	4 hours

presence of any *E. coli*, *Salmonella* spp, *Clostridium* spp, *S. aureus*, *B. cereus*, *Klebsiella* spp, and *Pseudomonas* spp is unacceptable and all other organisms should be below  $10^2$  cfu ml<sup>-1</sup>. This lack of agreement possibly stems from the diverse nature of enterally fed patients and their immune status, and the lack of information about the pathogenicity of microorganisms growing in enteral nutrient solutions. This latter topic will be discussed further in Section 1.6.1.2 and in Chapter Five.

In addition to the microbiological quality of enteral nutrient solutions, there are also guidelines which recommend the maximum length of time enteral nutrients should be held at room temperature for administration to the patient (hang time). Anderton *et al* (1986) recommend that non-sterile feeds (mixed-by-hand) should have a hang time of no more than 4 hours, while all other feed types and systems may be used for up to 24 hours. However, it is clear from the literature that there is vast range of clinical practice which varies from hospital to hospital. For example, Wagner *et al* (1994) report that the policy in their hospital is to limit hang times of mixed-by-hand and ready-to-use formula in open systems to 12 hours. This is considerably in excess of the guidelines of Anderton and co-workers (1986). Similarly, a number of authors recommend hang times of greater than 24 hours for "closed" feeding systems (Weenk *et al*, 1995; Wagner *et al*, 1994; Dentinger *et al*, 1995). Anderton *et al* (1986) also recommend a maximum hang-time for sip feeds of 4 hours. However, it difficult to believe that this is adhered to, especially when sip feeding is carried out in the home. This may again be as a result of deficiencies in the literature. Understanding of microbial growth in enteral nutrient solutions is poor. Few reports have used the newer types of enteral feeds on the market and those in existence are often weakened by a lack of kinetic data (lag, exponential, stationary phases and doubling times) and statistical credibility.

In summary, it is clear that there are no universally accepted standards for the microbiological quality of enteral nutrient solutions or the time they should be exposed to room temperature in the ward and home. Furthermore, microbiological standards for



enteral formulas are aimed at the prevention of exogenous contamination and do not take into consideration the problem of contamination from endogenous sources, which is more difficult to measure.

#### 1.5.1.2 Use of closed enteral feeding systems

The potential for "closed" enteral feeding systems to reduce microbial contamination from exogenous sources is evident. Microorganisms from contaminated ingredients and feed preparation equipment are eliminated because "closed" systems utilize sterile ready-to-use formulas. In addition, the risk of contamination arising during administration of the feed is reduced because "closed" feeding systems require less manipulation and no decanting of product is required. However, there is considerable debate about whether use of "closed" enteral feeding systems is effective as a means of excluding microorganisms, even though they are heavily promoted as such by manufacturers.

In recent times the benefits of "closed" feeding systems have been investigated in a number of studies. Wagner *et al*, (1994), Dentinger *et al*, (1995) and Weenk *et al* (1995) found that "closed" systems could remain almost entirely free from microbial contamination throughout hang times of 36, 48 and 48 hours respectively. However, there are significant weaknesses in the studies of Wagner *et al* (1994) and Dentinger *et al* (1995). The study of Wagner and co-workers (1994) sampled from the reservoir and therefore would not detect any contamination from endogenous sources which might be present in the feed tubing. Payne-James *et al* (1992) found that endogenous microbial contamination, ascending from the lower GI-tract, does not generally enter the feed reservoir. The study of Dentinger *et al* (1995) was conducted in the laboratory, with enteral nutrient solution flowing through the feeding system into a bucket. Therefore, there was no possibility of endogenous contamination arising.

In contrast to these studies, Crocker *et al* (1986), Donius (1993) and Chan *et al* (1994) concluded that levels of microbial contamination in "closed" systems are not significantly

better than in "open" systems. For example, Chan *et al* (1994) found that after 24 hours at ward temperature, there were concentrations of microorganisms between  $10^2$ - $10^7$  cfu ml<sup>-1</sup> in the distal end of a feeding tube from a closed enteral feeding system. This author and co-workers suggested that such high levels of contamination may have been of exogenous origin (entering the Y-port during flushing) or endogenous origin (ascending from the lower GI-tract. However, Donius (1993) found that the use of Y-ports in "closed" enteral feeding systems actually reduced the incidence and levels of microorganisms in the feed, indicating that Chan *et al* (1994) were probably detecting endogenous contamination.

Whereas "closed" systems may eliminate many of the routes through which exogenous contamination may arise, such systems are ineffective at preventing the colonisation of feed tubes by microorganisms ascending from the lower GI-tract. If "closed" systems are preferable to "open" systems, it is evident that they should not be used for extended hang times as recommended by Dentinger *et al* (1995) and Weenk *et al* (1995). It is clear from these studies that the benefits of "closed" enteral feeding systems should be investigated further, with specific reference to measurements of endogenous contamination.

#### **1.5.2 Current methods of chemically preserving enteral nutrient solutions**

Attempts have also been made to inhibit the growth of microorganisms in enteral nutrient solutions by means of chemical preservation with potassium sorbate, with varying degrees of success. Fagerman and co-workers, (1984) and Paauw and co-workers, (1984), found that 0.036-0.2% potassium sorbate was effective at inhibiting the growth of *Ent. cloacae* and suggested that this agent might have wider use in the preservation of enteral nutrient solutions. In contrast, Anderton (1985) found that the concentration of potassium sorbate present in an elemental type feed was insufficient to prevent the growth of *P. aeruginosa* at 25°C and *P. aeruginosa*, *Ent. cloacae* and *K. pneumoniae* at 37°C. Furthermore, potassium sorbate has a low pKa value (Scott and Gorman, 1992) and is therefore unsuitable for the preservation of milk-based enteral nutrient solutions

which have a neutral pH. However, considering the evidence which has been accumulated demonstrates both the inadequacies of the present provision for preventing contamination of enteral nutrient solutions, and the consequences for the patient when contamination occurs, it is surprising that more effective means of chemically preserving enteral nutrient solutions have not been investigated.

### **1.5.3 Summary**

Although methods that aim to exclude microorganisms from enteral feeding systems have had some success at preventing exogenous contamination, it is evident that they are ineffective against endogenous contamination. Chemical preservation of enteral feeds presents a possible method of inhibiting the growth of microorganisms which has the potential to be effective against endogenous contamination. However, as yet there are no effective preservatives which are used in enteral nutrient solutions or other milk-based products.

## **1.6 NOVEL STRATEGIES FOR PREVENTING INFECTIOUS COMPLICATIONS IN ENTERALLY FED PATIENTS BASED ON CHEMICAL PRESERVATION OF ENTERAL NUTRIENT SOLUTIONS**

The ability of microorganisms to cause disease is dependent on both host and microbial factors. A host that is young or very old; malnourished; receiving steroids or cytotoxic drugs; or has an impaired immune system as a result of disease (e.g. leukaemia or AIDS) is more vulnerable to microbial infection (Sleigh and Timbury, 1990). In addition, changes in the host microbial flora, as a result of broad-spectrum antibiotic treatment, may allow pathogens to become established in the host.

A microorganism's potential to cause disease is also dependent on its pathogenicity or virulence. The pathogenicity of an organism is a function of five requirements: infection of mucosal surfaces, entry into the host tissues through these surfaces; interference and evasion of host defences; multiplication in the *in-vivo* environment; and damage to the host (Smith, 1995). Each microbial strain produces a variety of cell surface structures, intracellular components, and extracellular products which define its pathogenicity. These may or may not confer the ability to cause disease in a host. The vast majority of enterally fed patients are considered to be immunocompromised to some degree and are thus more vulnerable to infection, even with microorganisms of relatively low pathogenicity (Anderton, 1993).

The use of novel chemical preservatives has the potential to reduce the risk of infectious complications in the enterally fed patient not only by inhibiting microbial growth but by inhibition or modification of virulence determinants. Chemical preservatives with this capability have the benefit of reducing the risk immunopathological responses to the uninhibited microbial population or to microbial components released during chemical inactivation. In addition, they also may inhibit the production of toxins and microbial defences. Selection of such chemical agents for use in enteral nutrient solutions must be made carefully, taking into account the types and properties of contaminating

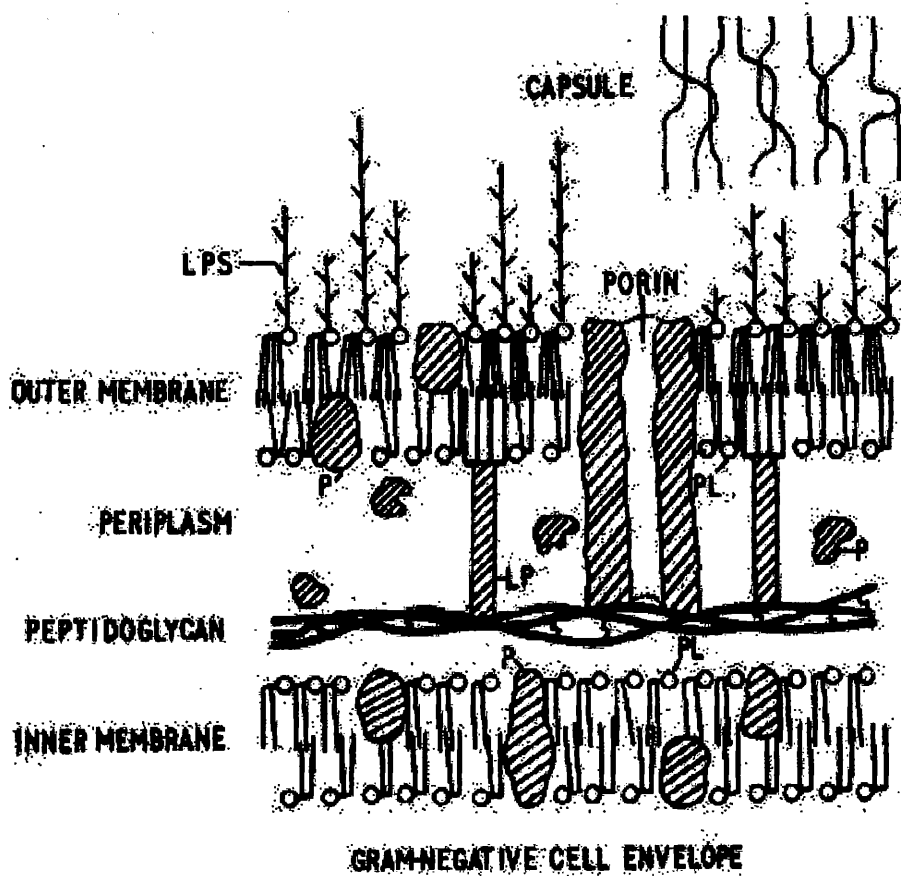
microorganisms, the composition of enteral nutrient solutions and the properties of the antimicrobial agent itself.

### **1.6.1 Criteria for selection of chemical preservatives suitable for use in enteral nutrient solutions**

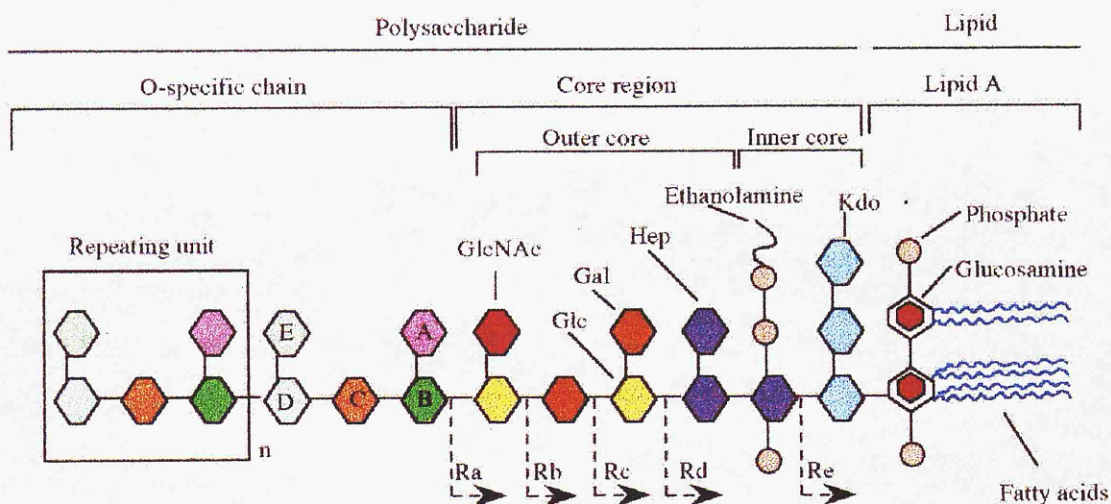
#### **1.6.1.1 Types and properties of contaminating microorganisms**

The microorganisms which contaminate enteral nutrient solutions are mainly Gram-negative bacteria (Navajas *et al*, 1992). Gram-negative bacteria have a high degree of intrinsic resistance to antimicrobial agents, conferred to them by the complex nature of their cell envelope and their ability to acquire resistance through genetic transference. (Hugo and Russell, 1992). Therefore, a selected antimicrobial agent must have a mode of action which overcomes the resistance provided by the cell envelope and there must be little or no genetic resistance to the antimicrobial agent in the microbial population. Gram-negative bacteria also have a vast number of products and structures which contribute to their pathogenicity.

Lipopolysaccharide is perhaps the most studied of all Gram-negative structures. Covering most of the outer surface of the bacterial cell it contributes both to bacterial antimicrobial resistance and pathogenicity (Figure 1.3). Lipopolysaccharide has been shown to have a role in the pathogenesis of Gram-negative sepsis resulting from bacteraemias and septicaemia (Morrison and Ryan, 1987; Kohn and Kung, 1995), and in some forms of respiratory infection (Kronborg, 1995). This thesis will focus on the lipopolysaccharides of the enterobacteriaceae (*E. coli*, *Enterobacter* spp. and *Klebsiella* spp.) and *Pseudomonas* spp which are particularly frequent contaminants of enteral nutrient solutions (Anderton, 1985). Each LPS molecule can be divided into three distinct regions: lipid A, core oligosaccharide and O-polysaccharide (Figure 1.4).



**Figure 1.3:** The outer membrane of Gram-negative bacteria. LPS: lipopolysaccharide; PL: phospholipid; P: protein. Adapted from Hancock and Poxton (1988).

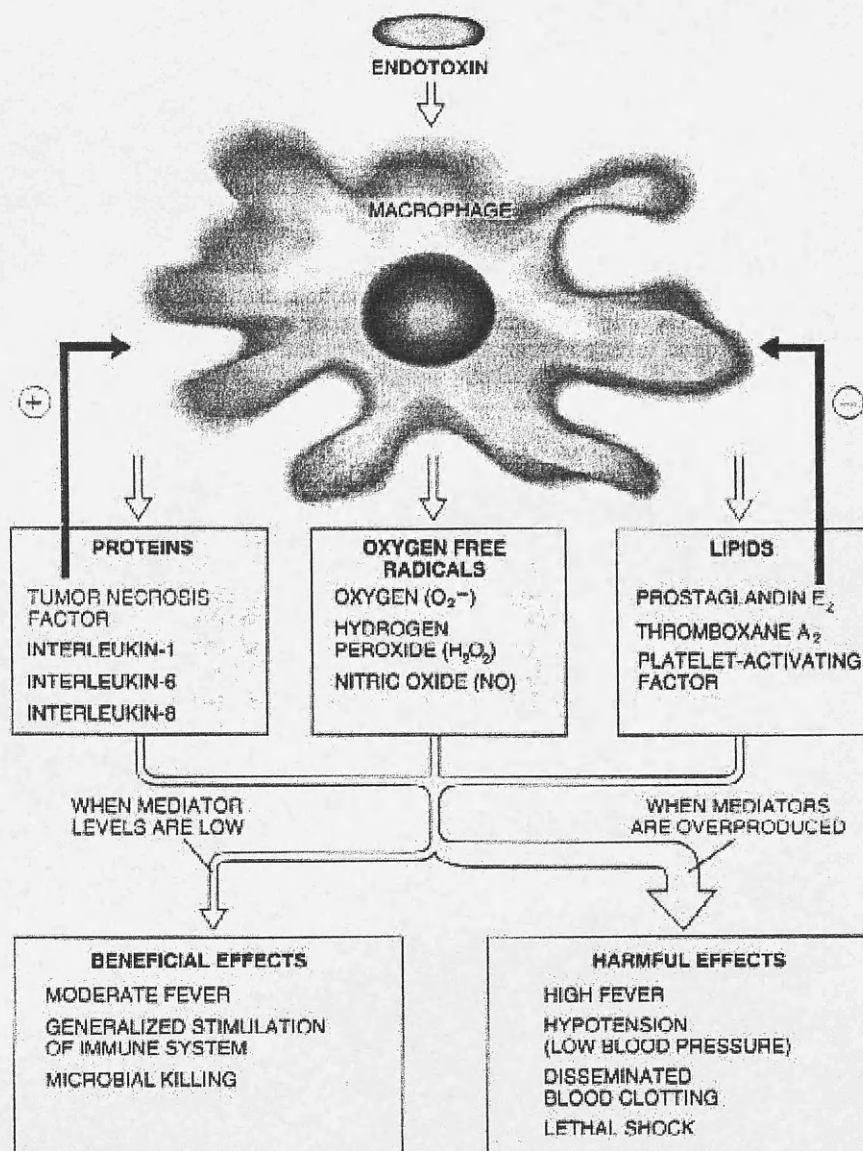


**Figure 1.4:** The chemical structure of *Salmonella typhimurium* lipopolysaccharide showing: A-E sugar residues; Glc: D-glucose; Gal: D-galactose; GlcNAc: N-acetyl-D-glucosamine; Hep: L-glycero-D-manno heptose; KDO: 2-keto-3-deoxyoctonic acid; Ra-Re are incomplete R-form lipopolysaccharides. Adapted from Rietschel and Brade (1992) by Urayet (1996).

Lipid A is a disaccharide, composed of D-glucosamines or 2,3-diamino-2,3-deoxy-D-glucose, substituted with phosphate groups and fatty acids (Proctor *et al*, 1995). The planar structure of lipid A enables LPS molecules to pack tightly together, anchoring the LPS to outer membrane and preventing the free movement of hydrophobic molecules across the Gram-negative cell envelope (Poxton, 1993). Although the basic structure of lipid A is conserved even across diverse genera of Gram-negative bacteria, variation does occur in the substitution patterns of phosphatyl groups and fatty acid chain length (Mutharia *et al*, 1984). These variations can have a large effect on the endotoxicity of the molecule (Poxton, 1993; Proctor *et al*, 1995).

Lipid A is responsible for virtually all the endotoxic properties of lipopolysaccharide, being a potent activator of macrophages (Morrison and Ryan, 1987). Small amounts of LPS generate pain, swelling and fever but have some beneficial effects to the host: enhancing antibody production; inducing the release of acute phase reactants; priming phagocytes for more effective bactericidal activity; and enhancing the tumoricidal activities of macrophages (Proctor *et al*, 1995 citing Adams, 1992). In contrast, the large amounts of LPS released in serious Gram-negative sepsis can result in shock and death (Rietschel and Brade, 1992). LPS is able to induce the release of large quantities of pro-inflammatory cytokines, especially Tumour necrosis factor (TNF $\alpha$ ), Interleukin (IL-1 $\alpha$ ) and IL-6, reactive oxygen species and bioactive lipid from macrophages (Nathan *et al*, 1980; Morrison and Ryan, 1987; Figure 1.5). These cytokines can in turn trigger the release of other cytokine cascades, bioactive lipids and reactive oxygen species such as nitric oxide, from other types of cells (Morrison and Ryan, 1987). This initiates a further release of messenger molecules (known as the mediator storm), causing increased vascular permeability, decreased cardiac output, vasodilation, pulmonary hypotension and disseminated vascular coagulation, ultimately resulting in shock and death. The release of early cytokines (TNF $\alpha$ , IL-1 $\alpha$  and IL-6) from macrophages has been shown to be accountable for almost all the symptoms of endotoxic shock syndrome (Freudenberg *et al*, 1986).



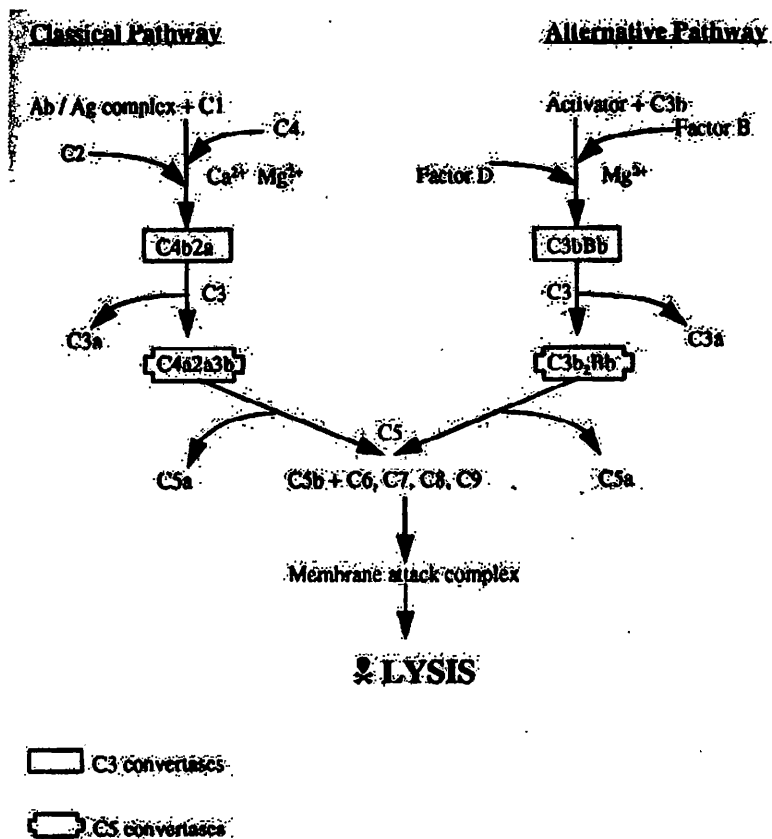


**Figure 1.5:** Release of inflammatory mediators from macrophages in response to lipopolysaccharide (endotoxin). Taken from Rietschel and Brade (1992).

Core oligosaccharide is generally sub-divided into the inner core and outer core regions. The inner core consists of 3-oxy-D-manno-2-octosonnate (KDO) attached to heptoses (mainly, L-glycero-D-mannose), substituted with phosphatyl and phosphoethanolamine groups. In common with lipid A, the inner core is highly conserved across different genera of Gram-negative bacteria. Mutations which leave an organism unable to synthesise KDO in the inner core are lethal, emphasising the importance of this part of the LPS molecule (and LPS as a whole) to the growth and viability of Gram-negative organisms (Rietschel and Brade, 1992). The outer core region is a more variable structure, consisting of hexoses such as glucose, galactose and N-acetylglucosamines. It is the outer core which mediates the binding of LPS or bacteria to the lectin-like receptors on T lymphocytes (Lehman *et al*, 1980).

O-polysaccharide consists of long chains of repeating oligosaccharide units, with each unit consisting of three to eight different or identical hexoses. Variation occurs in the number of units attached to each LPS molecule and the number and composition of each oligosaccharide unit, making O-polysaccharide the most variable part of LPS. Lipopolysaccharide which has O-polysaccharide attached is designated smooth LPS, while LPS molecules lacking O-polysaccharide is called rough LPS. This relates to the "crinkly" colony morphology produced by mutant strains unable to synthesize smooth LPS, as opposed to the smooth colony type of strains that produce smooth LPS. However, even in strains able to synthesize smooth LPS, the majority of the microbial cell surface is covered by rough LPS molecules (Poxton, 1993).

O-polysaccharide is known to have a number of important biological functions. Firstly, it increases cell-surface hydrophilicity which, in the absence of encapsulation, is important in preventing phagocytosis (Cunningham *et al*, 1975). It is also important in conferring serum resistance to Gram-negative bacteria. O-polysaccharide masks lipid A which would otherwise activate the classical complement pathway (Figure 1.6) in the absence of antibody (Morrison and Kline, 1977) and despite activating the alternative pathway



**Figure 1.6:** The classical and alternative pathways for the activation of the complement system (Adapted from Murray, 1994).

(Figure 1.6) it prevents lysis by preferentially binding the C3b component to long chain O-polysaccharide (Joiner *et al*, 1984; Grossman *et al*, 1987). This ensures that there is a sufficient distance to avoid insertion of the membrane attack complex (C5b-9) into the bacterial outer membrane (Joiner *et al*, 1984; Frank *et al*, 1987). Both length of O-polysaccharide chains and coverage of the lipid A/core are important in this respect (Goldman *et al*, 1984).

The variability of O-polysaccharide structure and composition also increases the ability of Gram-negative bacteria to evade specific immune responses. The attachment of specific antibodies to bacteria surfaces enhances phagocytosis by promoting attachment to Fc and complement receptors on the phagocyte surface activating the classical complement pathway and decreasing the hydrophobicity of the cell surface (Silverstein, *et al*, 1977). Activation of the classical complement pathway in this manner will also increase the rate of bacterial lysis and increase the inflammatory response (Frank *et al*, 1987). However, the huge variety of O-antigenic types confers an advantage to the bacterium because the host may have no immunological memory of it, thereby frustrating these mechanisms. Finally, O-polysaccharide increases resistance of Gram-negative bacteria to bile salts, heavy metals, antibiotics and some preservatives (Proctor *et al*, 1995; Hugo and Russell, 1992).

Therefore, an important aspect of selecting a suitable antimicrobial agent for preserving enteral nutrient solutions is to assess its ability to inhibit Gram-negative bacteria and modify the structure and immunological properties of LPS.

#### 1.6.1.2 The properties of the enteral nutrient solution

The composition of enteral nutrient solutions is unfavourable to the activity of many antimicrobial agents because there are high levels of proteins, lipids and carbohydrates which have the potential to inactivate or bind to the preservative. Enteral nutrient

solutions are also emulsions in which hydrophobic antimicrobial agent may partition into the lipid phase away from growing microorganisms.

In addition, the effect of the enteral nutrient solutions on the pathogenicity of the microorganisms growing in them should be considered. This is because many microorganisms, including Gram-negative bacteria, show phenotypic variation of their virulence factors in response to environmental signals such as temperature, pH, osmolarity and nutrient limitation (Kroll, 1990). Studies in laboratory media has shown that nutrient limitation or supplement can affect the expression of Gram-negative virulence determinants such as capsules (Mengistu *et al*, 1994), fimbriae (McGee *et al*, 1979), extracellular proteins (Sokol and Woods, 1984; Mouriño *et al*, 1994; Ebel *et al*, 1996), lipopolysaccharide (Kelly *et al*, 1989; Nelson *et al*, 1991; Allan and Poxton, 1994) and outer membrane proteins (Darveau *et al*, 1983; Brown and Williams, 1985; Camprubi *et al*, 1992; Allan and Poxton, 1994). Therefore, the pathogenicity of Gram-negative bacteria growing in enteral nutrient solutions may be radically different from our current understanding based on studies conducted in laboratory media. To date, there are no characterization studies of Gram-negative bacterial virulence in enteral nutrient solutions and only one report characterizing a virulence factor of Gram-positive bacterial species (Rowan *et al*, 1997). If attempts are to be made to alter microbial virulence in enteral nutrient solutions, accurate characterization of virulence factors is essential.

#### **1.6.1.3 Properties of the antimicrobial agent**

Antimicrobial agents should also be free from toxicity problems, must not have adverse organoleptic properties and should be stable during heat processing.

#### **1.6.2 Potential antimicrobial agents for use as preservatives in enteral nutrient solutions**

In the light of the criteria for selecting antimicrobial agents suitable for use in enteral feeding systems, there is a considerable variety of chemical preservatives which may be

considered for inclusion in the formulation of enteral nutrient solutions. These include preservatives already available on the market, such as nisin and parabens, or novel preservatives. This latter group includes antimicrobial agents of animal origin such as lactoperoxidase and lysozyme (Beuchat and Golden, 1989); of bacterial origin such as carnocin 54 (Schillinger *et al*, 1995) and plantaricin SA6 (Rekhif *et al*, 1995); and of botanical origin such as polyphenols (Cappasso *et al*, 1995), humolones, and essential oil (Nychas, 1995). The use of essential oils are of interest in this thesis.

#### 1.6.2.1 Essential oils

Essential oils are derived from the steam distillation or cold-pressing of a single botanical species (Beuchat, 1994 citing Farrell, 1990). They are a mixture of volatile organic compounds which contribute to the flavour and fragrance of the plant from which they have been extracted (Beuchat, 1994 citing Farrell, 1990). They may be extracted from the flowers, leaves, seeds, stems, roots, rhizomes, woods, barks, bulbs and buds of plants (Shelef, 1983) and are usually pale or colourless liquids, although a minority are highly coloured. Essential oils are composed of hundreds of individual components which are odoriferous and often have biological activity. The composition of many essential oils is unknown and therefore the biological activity of these components also remains to be investigated. However, over thousands of years man has utilized the biological activity of essential oils and the plants from which they come, in the preparation of foods, perfumes, medicines and therapies. In recent times their properties have come under renewed scrutiny as the public have demanded "natural" alternatives to existing conventional drugs and therapies. Various essential oils have been shown to have anaesthetic (Watts and Peterson, 1987), anti-inflammatory (Pulla-Reddy and Lokesh, 1994), antioxidant, (Anderton *et al*, 1996), antispasmodic (Reiter and Brandt, 1985; Kingham, 1995) and antimicrobial properties (Deans and Richie, 1987; Nychas, 1995). It is the antimicrobial properties of essential oils that are of interest in the context of preserving enteral feeds.

Essential oils have several characteristics which may make them suitable for use as antimicrobial agents in enteral feeds. Firstly, they are perceived as "natural" by the public, are stable in foods (Juven *et al*, 1994) and have no legal constraints against their use in foods (The Preservative in Foods Regulations, 1989). Secondly, bacteria are unlikely to have intrinsic resistance to them because each oil consists of a large number of components which are likely to have several modes of action, thus decreasing the likelihood of resistance being developed to the oils. Finally, a number of essential oils have been shown to inhibit the production of microbial toxins (Bullerman, 1974; de Witt *et al*, 1979) and although research in this area is limited it is possible that essential oils may alter other virulence factors as well.

#### 1.6.2.2 The preservation of foods using essential oils

Interest in essential oils as food preservatives has arisen out of the ancient tradition that spices and herbs, from which essential oils are extracted, have functional properties other than that of flavour. Subsequent scientific studies in laboratory media have demonstrated that ground or kibbled spices have antimicrobial activity against important food pathogens such as *E. coli*, *Listeria monocytogenes*, *S. typhimurium*, *S. aureus* and *Vibrio parahaemolyticus* (Johnson and Vaughn, 1969; Aktug and Karapinar, 1986; Evert-Ting and Deibel, 1992; Hefnawy *et al*, 1993). However, authors trying to reproduce these results in food have found that whole herbs and spices either had no antimicrobial effect, or the amounts of herbs and spices required to inhibit the microbes made the food unpalatable (Shelef *et al*, 1984). Furthermore, some investigations found that the magnesium content of plant material stimulated the metabolic functions of some bacteria (Zaika and Kissinger, 1981; Zaika and Kissinger, 1984). Other studies indicate that because most herbs and spices are grossly contaminated with bacteria they contribute more microorganisms to the food than they inhibit (Shelef, 1983).

The use of essential oils in foods has the potential to overcome the problems associated with whole herbs and spices. They are sterile, do not contain magnesium and have a

much higher concentration of active molecules. A wide range of essential oils have been investigated for their potential as antimicrobial agents. Investigations in laboratory media have shown that many of them have wide-spectrum inhibitory activity against bacteria, yeast and fungi (Table 1.3). However, some of the data is contradictory and it is impossible to make objective comparisons between studies because of the diversity in the methods and conditions used. Despite these deficiencies in the existing literature, the essential oils of *Allium* spp. (Garlic, onion and leek), bay, cinnamon, clove, oregano, rosemary, sage and thyme are generally considered the most inhibitory essential oils that are suitable for use in foods (Beuchat, 1994; Nychas, 1995).

Unfortunately, studies attempting to utilize the antimicrobial properties of essential oils in foods have found the concentrations required to effect protection against growth of pathogens overwhelm the flavour of most foods in an unacceptable manner (Shelef, 1983; Shelef *et al*, 1984; Nychas, 1995). As a result, few suitable applications in foods have been found for the antimicrobial properties of essential oils. However, the bland nature of enteral nutrient solutions may make them a suitable application for a preservative which has a strong and distinct flavour (Davidson and Richardson, 1996).

#### 1.6.2.3 Active components of essential oils

The majority of constituents in essential oils are either phenolics or terpenes (Max, 1992). Most authors are of the opinion that it is the phenolic compounds in essential oils that are responsible for the antimicrobial activity (Shelef, 1983; Beuchat and Golden, 1989; Nychas, 1995). Katayama and Nagai (1960) cited by Nychas (1995) concluded that the active components of essential oils were eugenol, carvacrol, isoborneol, thymol, vanillin and salicylaldehyde. These are all phenolic molecules. This was in agreement with work of Oka (1960) who found thymol to be active, and the work of Karapinar and Aktug (1987) who found that anethole, thymol and eugenol were particularly active. More detailed studies by Deans and Svodboda (1988) and Deans and Svodboda (1989) found that some terpenes also have activity and many of the minor constituents in



**Table 1.3: Essential oils with antimicrobial activity.**

<b>Achoite</b>	<b>Cocoa</b>	<b>Lovage</b>	<b>Sage</b>
<b>Allspice</b>	<b>Coffee</b>	<b>Mace</b>	<b>Sassafras</b>
<b>Almond (Bitter)</b>	<b>Coriander</b>	<b>Mandarin</b>	<b>Summer Savory</b>
<b>Angelica</b>	<b>Dill</b>	<b>Majoram</b>	<b>Winter Savory</b>
<b>Anise</b>	<b>Elecampane</b>	<b>Musky bugle</b>	<b>Spearmint</b>
<b>Basil</b>	<b>Eucalyptus</b>	<b>Mustard</b>	<b>Star anise</b>
<b>Bay</b>	<b>Fennel</b>	<b>Nutmeg</b>	<b>Tarragon</b>
<b>Bergamot</b>	<b>Fenugreek</b>	<b>Onion</b>	<b>Tea</b>
<b>Calmus</b>	<b>Garlic</b>	<b>Orange</b>	<b>Tea Tree</b>
<b>Cananga</b>	<b>Geranium</b>	<b>Oregano</b>	<b>Thyme</b>
<b>Caraway</b>	<b>Ginger</b>	<b>Paprika</b>	<b>Turmeric</b>
<b>Cardamom</b>	<b>Horseradish</b>	<b>Parsley</b>	<b>Vanilla</b>
<b>Celery</b>	<b>Leek</b>	<b>Pennyroyal</b>	<b>Verbena</b>
<b>Chenopodium</b>	<b>Lemon</b>	<b>Pepper</b>	<b>Wintergreen</b>
<b>Cinnamon</b>	<b>Lemongrass</b>	<b>Peppermint</b>	
<b>Citronella</b>	<b>Lime</b>	<b>Pimento</b>	
<b>Clove</b>	<b>Liquorice</b>	<b>Rosemary</b>	

Adapted from Beuchat (1994) citing Boonchird and Flegel (1982), Conner and Beuchat (1984 a, b), Deans and Richie (1987), Maruzella and Liquori (1958) and Huhtanen (1980). Emboldened names indicate those suitable for inclusion in foods.

essential oils make an overall contribution to the activity. It is clear that more research is required to isolate more of the numerous components of essential oils and measure their antimicrobial activity.

#### 1.6.2.4 The antimicrobial mode of action of essential oils

Although considerable research has occurred to establish the antimicrobial activity of essential oils, their precise mode of action remains unknown. However, as many of the inhibitory constituents of essential oils are phenolic, it is possible that the mode of action is similar to other phenolic compounds such as oleuropeins, butylated-hydroxy-anisole (BHA), parabens and phenol itself. There is consensus that these phenolics attack microbial cells not only by disrupting the cytoplasmic membrane and wall (thus releasing cell contents) but by causing membrane dysfunction (Nychas, 1995). This type of damage has been shown to affect electron transport, nutrient uptake, nucleic acid synthesis, and ATPase activity (Eklund, 1980; Degre and Sylvestre, 1983; Degre *et al*, 1983; Rico-Munoz *et al*, 1987; Ruiz-Barba *et al*, 1990; Denyer and Hugo, 1991; Hugo 1991a ; Kabara and Eklund, 1991; Eklund and Nes, 1991; and Martin, 1992). It is clear that phenolics do not have one single target on microbial cells, and it is unlikely that there is a common mode action which affects all microorganisms (Rico-Munoz *et al*, 1987; Nychas, 1995).

#### 1.6.2.5 The effect of essential oils on microbial virulence

Sub-lethal concentrations of conventional antibiotics are known to have a considerable impact on the virulence and pathogenicity of microorganisms. They are known to alter their morphology, growth rate, production of toxins, enzymes and antibiotic degrading substances and products released on bacterial death (Nelson *et al*, 1993; Lorian and Gemmell, 1991). This may change the capability of the microbes to adhere to tissues, evade opsonophagocytosis and to resist complement mediated lysis (Lorian and Gemmell, 1991). However, little is known about the effects of sub-lethal doses of essential oils and their components on the pathogenicity and virulence of most

microorganisms. This is not surprising given that the understanding of the mechanisms by which essential oils inhibit microorganisms is so poor. Even so, several authors have reported the anti-toxigenic properties of essential oils. De Witt and co-workers (1979) described the inhibition of toxin production by *Clostridium botulinum* type A in meat slurry containing 1500  $\mu\text{g g}^{-1}$  garlic or onion oil. Unfortunately, this same concentration of oils was unable to prevent toxin production by type B and E *C. botulinum* (de Witt *et al*, 1979). The inhibition of aflatoxin produced by *Aspergillus parasiticus* has also been reported in the literature. Bullerman (1974) found that 1-2% ground cinnamon in laboratory broth was sufficient to prevent aflatoxin production by *A. parasiticus*, but insufficient to prevent growth. Cinnamon oil was found to be effective at 200  $\mu\text{g ml}^{-1}$  while cinnamic aldehyde (a major component of cinnamon oil) was effective at 150  $\mu\text{g ml}^{-1}$  (Bullerman *et al* 1977). Similarly, these authors found that clove oil was an effective anti-aflatoxigenic at 250  $\mu\text{g ml}^{-1}$ , while eugenol, the main component of clove oil, had an inhibitory effect at 125  $\mu\text{g ml}^{-1}$ . Batt *et al*, (1983) found that carrot seed oil and most of its major components (geraniol, citral, limonene, terpinene, borneol acetate and terpineol) had anti-aflatoxigenic properties. It is possible that despite the strong olfactory and organoleptic properties of essential oils and their constituents, they have potential to be used at low levels to alter the pathogenicity of some microorganisms, especially by inhibiting toxin production.

#### 1.6.2.6 Combinations of preservatives containing essential oils

The potential usefulness of essential oils as food preservatives may be increased if they are part of a combination of preservatives. Combinations of preservatives have advantages over the use of single agents in that they are less likely to encounter microbial resistance and offer the possibility of antimicrobial synergism (Eliopoulous and Moellering, 1991). Synergy enables the reduction in the concentration of components used without any loss of activity. This may have the benefit of reducing the costs of preservation while simultaneously enabling the use of antimicrobial agents otherwise unpalatable in foods. Studies have examined the antimicrobial effects of combining spices

and their essential oils with salt (Anand and Johar, 1957; Shelef *et al*, 1984), sorbate (Mori *et al*, 1974) and sugar solutions (Briozzo *et al*, 1989). However, none of these authors ascribed synergistic antimicrobial properties to their combination preservatives.

### **1.6.3 Summary**

Desirable qualities of a chemical preservative for use in an enteral feeding system are the ability to inhibit the growth of microorganisms (especially Gram-negative bacteria) and to reduce the virulence of remaining organisms, without being toxic to the patient, being inhibited by constituents of the enteral feed or incurring rapid microbial resistance. Essential oils, used singly or in combination have the potential to fulfil a number of these qualities. In order to accurately study the potential of essential oils to alter virulence of microorganisms, virulence characteristics of contaminating organisms should be determined in the enteral formula.

## **1.7 AIMS OF THESIS**

Microbial contamination is a problem in enteral feeding systems because patients may become colonized with microorganisms and develop serious infections. Methods currently used to either exclude microbial contamination from the enteral feeding system or inhibit the growth of these microorganisms seem to be ineffective, especially against contamination from endogenous sources. Plant essential oils have the potential to be used, singly or in combination with other preservatives, to inhibit the growth and reduce the pathogenicity of microorganisms that contaminate enteral feeding systems. Many aspects of microbial pathogenicity are dependent on environmental stimuli provided by the growth environment. Therefore, the pathogenicity of microorganisms growing in enteral nutrient solutions may be different to current understanding of the virulence which is based on experiments in laboratory media. In order to realize the potential of essential oils as useful antimicrobial agents in enteral feeding systems, the growth and pathogenicity of microorganisms in enteral nutrient solutions need to be investigated. Therefore the aims of this thesis are as follows:

- To investigate the growth of the types of microorganisms that are commonly isolated from enteral feeds.
- To measure the inhibitory properties of essential oils (oil of fennel) used singly or in combination with other chemical preservatives (parabens) against types of microorganisms that are commonly isolated from enteral feeds.
- To investigate the pathogenicity of microorganisms in enteral nutrient solutions and compare with laboratory media.
- To investigate the potential of essential oils (oil of fennel) used singly or in combination with other chemical preservatives (parabens) to alter the pathogenicity of microorganisms that are commonly isolated from enteral feeds.

## **CHAPTER 2**

### **GENERAL MATERIALS AND METHODS**

#### **2.1 MATERIALS**

##### **2.1.1 Microbial strains**

Microbial strains were either involved in documented cases of infections arising from the consumption of contaminated enteral nutrient solutions, or used by other authors in previous studies into contamination of enteral nutrient solutions (Anderton, 1985). *Candida albicans* NCTC 3179, *Escherichia coli* NCTC 8007 and *Klebsiella aerogenes* NCTC 8172 were purchased from the National Collection of Type Cultures (NCTC), Porton Down, Wiltshire, UK. *Enterobacter cloacae* ATCC 23355, *Pseudomonas aeruginosa* ATCC 9721 and *Staphylococcus aureus* ATCC 25923 were purchased from the American Type Culture Collection (ATCC), Rockville, Maryland, USA.

##### **2.1.2 Chemical suppliers**

Chemicals were purchased from either BDH, Merck House, Poole, Dorset, UK, Sigma Chemicals, Sigma-Aldrich, Fancy Road, Poole Dorset, UK or Gibco Limited, Paisley, Scotland. De-ionised water and pyrogen free (PF) water were produced using a Millipore Water system.

##### **2.1.3 Bacterial culture media**

###### **2.1.3.1 Standard Laboratory Media for Bacterial Culture**

Nutrient, typtone soya and Sabouraud broths and agars were purchased from Oxoid, Unipath Limited, Wade Road, Basingstoke, Hampshire, UK.

### 2.1.3.2 Enteral Nutrition Solutions

Osmolite and Pulmocare were kindly supplied in 250 ml cans by Ross Products Division, Abbott Laboratories Limited, Abbott House, Norden Road, Maidenhead, Berkshire, UK. Fortisip and Fortijuce were kindly supplied in 200 ml Tetra-Brik packs by Nutricia Clinical Care, Cow and Gate-Nutricia Limited, Whitehorse Business Park, Trowbridge, Wiltshire, UK.

## 2.2 METHODS

### 2.2.1 Maintenance of microbial strains

Cultures were maintained on nutrient, tryptone soya or Sabouraud agar plates, or in glycerol (BDH) stocks at -20°C. Nutrient broth and agar were used for the culture of *E. coli*, *Ent. cloacae*, *K. aerogenes* and *P. aeruginosa*, tryptone soya broth and agar was used for *S. aureus*, and Sabouraud broth and agar for the culture of *C. albicans*. Different growth media were used for the culture of *S. aureus* and *C. albicans* because nutrient broth and agar did not support sufficient growth of these organisms over a 24 hour period. Glycerol stocks were prepared by the method described by Fritsch and Sambrook, (1982). In summary, sterile broth (10 ml) was inoculated with a large colony of bacteria from stock culture plates (grown from a reconstituted freeze dried culture supplied by the culture collections) and incubated overnight at 37°C. 8.5 ml of overnight culture was taken, vortexed with sterile glycerol (1.9 g) and frozen at -20°C in 1 ml aliquots in sterile Eppendorf tubes.

Working stocks of each culture were prepared by inoculating well dried nutrient agar, tryptone or Sabouraud agar plates with the appropriate organism. Bacterial plates were incubated for 24 hour at 37°C and yeast plates at 25°C for 48 hours. Plates were stored at 4°C for up to 4 weeks before sub-culturing. Working stock cultures were renewed from a frozen glycerol stock culture every six months. Gram stains were performed at each stage to check the purity of the cultures.

### **2.2.2 Preparation of overnight cultures**

Bacterial overnight cultures ( $8-9 \log \text{ cfu ml}^{-1}$ ) were prepared by inoculating sterile nutrient broth or tryptone soya broth (10 or 200 ml) with a large colony from a bacterial stock culture plate and incubating at  $37^{\circ}\text{C}$  for 16-18 hours. Yeast overnight cultures ( $7-8 \log \text{ cfu ml}^{-1}$ ) were prepared by inoculating sterile Sabouraud broth (10 ml) with yeast stock culture and incubating at  $25^{\circ}\text{C}$  for 16-18 hours.

### **2.2.3 Viable counts**

Growth of microorganisms in broths or enteral nutrient solutions was measured by colony counts using the spread plate method (Jay, 1992). In summary, sterile laboratory media or enteral nutrient solutions were inoculated with overnight cultures prepared as outlined in Section 2.2.2 or with overnight culture serially diluted in 0.01M phosphate buffered saline (PBS), pH 7.4 (Sigma). Samples were removed at appropriate time intervals and serial tenfold dilutions were prepared in 0.01M PBS to give countable colonies when 0.1 ml was spread on agar plates. A mean value and standard error of the mean (SEM) were calculated from 3 replicate agar plates and expressed as  $\log \text{ cfu ml}^{-1}$ . Results with a SEM of less than 10% were considered acceptable. Each experiment was repeated three times and results were presented graphically as a mean of three independent experiments.



## CHAPTER THREE

# GROWTH OF MICROORGANISMS IN ENTERAL NUTRIENT SOLUTIONS

### 3.1 INTRODUCTION

Microbial growth rates in foods are determined by the initial inoculum or "load" of microorganisms present in the food, the storage temperature and conditions, (extrinsic parameters) and intrinsic parameters such as pH, osmolarity, water activity, nutrient availability and the presence of antimicrobial agents (Jay, 1992). Enteral nutrient solutions can be considered as foods designed to meet the specific needs of the malnourished and those unable to feed themselves through conventional methods (McCamish *et al*, 1997).

The extrinsic parameters affecting growth in enteral nutrient solutions vary considerably. Initial numbers of contaminating microorganisms may be virtually undetectable (Wagner *et al*, 1994) or as high as  $10^6$  cfu ml<sup>-1</sup> (Anderton, 1993). Microorganisms may interact with enteral nutrient solutions at temperatures which range from room temperature (22-25°C) to body temperature (37°C).

The intrinsic parameters which affect growth in enteral nutrient solutions also vary depending upon the type of feed used (milk-based, "fruit-based", elemental), the manufacturer and the precise purpose of the enteral product. A number of authors have considered enteral nutrient solutions as ideal environments for supporting rapid microbial growth (White *et al*, 1979; Simmons, 1981; Bastow *et al*, 1982). Others have shown that not all types of enteral feeds provide conditions conducive to microbial growth (Furtado *et al*, 1980; Hostetler *et al*, 1982; Stanek *et al*, 1983; Fagerman *et al*, 1984; Pauuw *et al*, 1984; Anderton, 1985; Oversen *et al*, 1991). This latter group of authors found that the growth of some microbial strains was inhibited in enteral nutrient solutions which had

low pH, high osmolarity, antimicrobial agents such as potassium sorbate, or combinations of these factors. For example, Anderton (1985) found that *E. coli*, *Ent. cloacae*, *K. aerogenes*, *S. aureus*, and *P. aeruginosa* grew well at 25°C and 37°C in two milk-based enteral nutrient solutions (Clinifeed ISO and Triosorbon), which had neutral pH, isomolarity with human plasma and contained no preservatives. Growth of *E. coli* and *S. aureus* in Vivonex HN, an enteral feed which has a low pH (pH 4.4), a high osmolarity (800 mOsL-1) and contains potassium sorbate, was inhibited at both temperatures, while growth of *Ent. cloacae* and *K. aerogenes* was inhibited at 25°C but not 37°C.

However, adverse conditions present in enteral nutrients do not seem to be effective at inhibiting the growth of all microorganisms. Anderton (1985) reported that *P. aeruginosa* grew in Vivonex HN at 25°C and 37°C despite the presence of unfavourable conditions. Even so, Oversen *et al* (1991) suggested that low pH and high osmolarity could be used to inhibit bacterial growth in enteral feeds. This was clearly contradicted by the work of Anderton (1985). Similarly, Fagerman *et al* (1984) and Pauuw *et al* (1984) suggested that potassium sorbate, a fungistatic ingredient of some foods, could be added to enteral nutrient solutions as a bacteristatic agent, because it inhibited *Ent. cloacae* at a concentration of 0.036% in Travasorb. However, Vivonex HN contains potassium sorbate and this proved to be ineffective against *Ent. cloacae* and *K. aerogenes* at 37°C and *P. aeruginosa* at 25°C and 37°C in the study of Anderton (1985).

Despite the existing literature, our knowledge of microbial growth in enteral nutrient solutions is poor. Many of the studies lack important kinetic data such as lag, exponential and stationary phase times and mean generation times (MGT). In addition, some of these studies have inadequate numbers of time points and poor statistical scrutiny. There is also some conflict between the conclusions of some studies. This is perhaps as a result of the range of products tested, the strains of organisms used in studies and other variations

in experimental conditions. Knowledge of microbial growth in enteral nutrient solutions is important for several reasons. Firstly, it is required to provide a scientific basis for the development of clinical practice in enteral feeding such as determining "hang"-times. Secondly, it is important for developing methodologies which investigate the virulence of microorganisms growing in enteral nutrient solutions. Finally, it also important for microbial inhibition studies in enteral nutrient solutions. Therefore, the aims of this study are to:-

- Measure the kinetic parameters of microbial growth, such as lag, exponential, stationary phases and mean generation times.
- Investigate those factors affecting microbial growth in enteral nutrient solutions, which are pertinent to improving enteral feeding practice.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Materials

The microbial strains used in experiments are listed in Section 2.1.1 and the chemicals and growth media are listed in Sections 2.1.2 and 2.1.3 respectively.

### 3.2.2 Methods

#### 3.2.2.1 Microbial enumeration methods

10 ml aliquots of sterile laboratory media or enteral nutrient solutions in glass test-tubes were inoculated with 10  $\mu$ l of overnight cultures (Section 2.2.2) or overnight cultures serially diluted in 0.01 M PBS. This gave initial microbial concentrations approximately in the range 2.00-5.00 Log cfu ml<sup>-1</sup>. Viable counts were performed over a 24 hour period using the procedure described in Section 2.2.3. Results were represented graphically and Mean Generation Times were calculated using the method described by Stanier *et al*, (1977). Lag, exponential and stationary phases in the growth were determined using the definitions of Atlas, (1995). The lag phase was defined as *"the period of time at the beginning of the bacterial growth cycle when there is little increase in bacterial numbers."* Student t-tests were used to determine at which time point changes in bacterial numbers became significantly more than the initial microbial concentration, thus quantifying the lag phase. The exponential phase was defined as *"the period of time in which cell numbers increase exponentially with growth."* Finally, the stationary phase was defined as *"the period of time after exponential growth when there is no further net increase in bacterial numbers."* The definition of diauxic growth was that of Lewis and co-workers (1993). Students t-tests were used to determine the length and presence of this phase in the same manner as for the lag phases. Growth in different growth conditions was compared and analysed using Analysis of Variance (ANOVA) and student t-tests.

### 3.2.2.2 pH Measurements

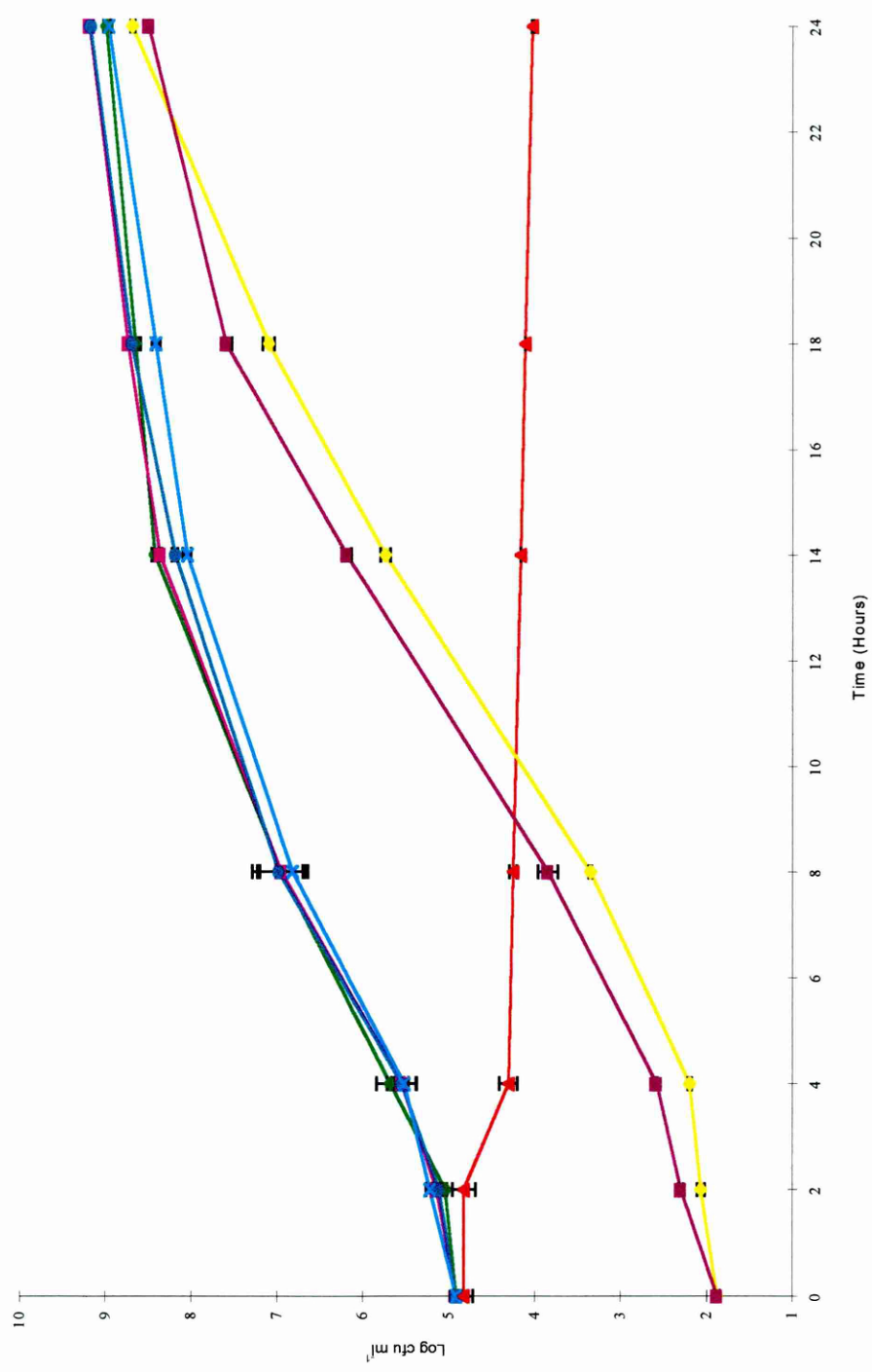
pH was measured using a Griffin Digital pH meter and probe (Griffin and George Limited, Loughborough, Leicestershire, UK). The meter was calibrated before use with pH 4 and pH 7 standard buffer solutions (BDH). Results were expressed graphically as the mean of three independent readings with SEM. pH readings from growth of *K. aerogenes* in different enteral nutrient solutions and nutrient broth were analysed using ANOVA and students t-tests.

### 3.3 RESULTS

#### 3.3.1 Growth of *E. coli* in nutrient broth and enteral nutrient solutions

Nutrient broth, Osmolite, Fortisip, Pulmocare and Fortijuice were inoculated with two inocula sizes of *E. coli* and viable counts were made over 24 hours (Figure 3.1). At the higher inoculum, and in contrast to nutrient broth and milk-based enteral nutrient solutions, there was no growth in Fortijuice, numbers decreasing to  $4.04 \log \text{cfu ml}^{-1}$  over 24 hours. The growth curve of *E. coli* in Fortijuice was found to be significantly different ( $p < 0.001$ ) from that in nutrient broth. However, this was not true of the growth curves of *E. coli* in milk-based enteral nutrient solutions where numbers increased considerably. No significant differences were observed in the growth of *E. coli* over 24 hours in nutrient broth and milk-based enteral nutrient solutions (Osmolite, Fortisip and Pulmocare). There were 2 hour lag phases in the growth of *E. coli* between nutrient broth and Fortisip, but not Osmolite or Pulmocare. Growth occurred in nutrient broth and milk-based enteral nutrient solutions in two phases. The first phase consisted of relatively fast growth and lasted until 14 hours, while during the second phase growth was much slower (Table 3.1). By the end of the experiment *E. coli* numbers had increased to  $9.00 \log \text{cfu ml}^{-1}$  in nutrient broth,  $9.20 \log \text{cfu ml}^{-1}$  in Osmolite,  $9.19 \log \text{cfu ml}^{-1}$  in Fortisip and  $8.98 \log \text{cfu ml}^{-1}$  in Pulmocare. Growth in Osmolite and Fortisip was found to be significantly more ( $p < 0.05$  and  $p < 0.05$  respectively) than in nutrient broth, while growth in Pulmocare was not significantly different.

The results obtained using the lower inoculum of *E. coli* were similar to those observed using the higher inoculum. However, the growth curve of *E. coli* in Osmolite was significantly different ( $P < 0.001$ ) from that in nutrient broth. There were no lag phases in either medium but growth was slow for the first 4 hours in nutrient broth. After this period, the growth rate increased considerably so that the bacterial numbers in nutrient broth had increased from  $2.20 \log \text{cfu ml}^{-1}$  to  $8.69 \log \text{cfu ml}^{-1}$  by the end of the experiment. In contrast to this growth pattern, the growth of *E. coli* in Osmolite was fast



**Figure 3.1:** Growth of *Escherichia coli* in nutrient broth and enteral nutrient solutions. \*Each point represents the mean of three independent experiments with its SEM. ♦ Each point represents a mean of three plate counts with its SEM. \*Green: Nutrient broth, high inoculum; \*Magenta: Osmolite, high inoculum; \*Dark cyan: Fortisip, high inoculum; \*Cyan: Pulmocare, high inoculum; \*Red: Fortisip, high inoculum; ♦ Yellow: Nutrient broth, low inoculum; ♦ Purple: Osmolite, low inoculum.

**Table 3.1** Mean Generation Times for *Escherichia coli* growing in nutrient broth, Osmolite, Fortisip, Pulmocare and Fortijuce.

Inoculum Size	Growth Medium	Growth Phase 1	Growth Phase 2
<b>5.00 log cfu ml<sup>-1</sup></b>	<b>nutrient broth</b>	44 min (2-14 hours)	219 min (14-24 hours)
	<b>Osmolite</b>	73 min (0-14 hours)	151 min (14-24 hours)
	<b>Fortisip</b>	53 min (2-14 hours)	126 min (14-24 hours)
	<b>Pulmocare</b>	56 min (0-14 hours)	136 min (14-24 hours)
	<b>Fortijuce</b>	N/G	
<b>2.00 log cfu ml<sup>-1</sup></b>	<b>nutrient broth</b>	226 min (0-4 hours)	56 min (4-24 hours)
	<b>Osmolite</b>	57 min (0-18 hours)	120 min (18-24 hours)

N/G No growth of organism in medium.

N/A Not applicable.

( ) Duration of growth phase.

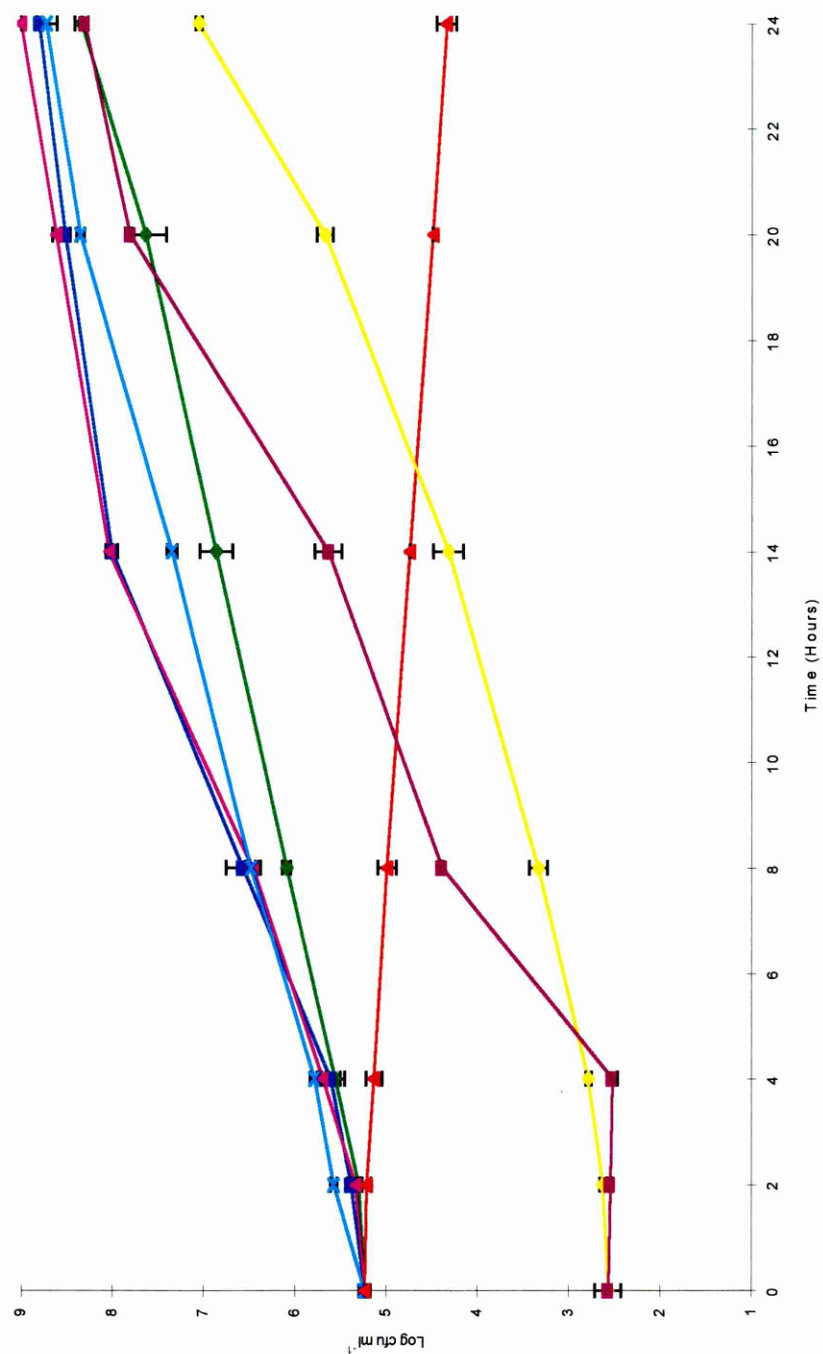


for the first 18 hours with numbers increasing from 1.88 log cfu ml<sup>-1</sup> to 7.11 log cfu ml<sup>-1</sup>. The growth rate slowed after 18 hours with the *E. coli* concentration being 8.50 log cfu ml<sup>-1</sup> at the end of the experiment. There was no significant difference between numbers of *E. coli* in nutrient broth and Osmolite at the end of the experiment.

### **3.3.2 Growth of *Ent. cloacae* in nutrient broth and enteral nutrient solutions**

Nutrient broth, Osmolite, Fortisip, Pulmocare and Fortijuce were inoculated with two inocula sizes of *Ent. cloacae* and viable counts were made over 24 hours (Figure 3.2). At the high inoculum (approximately 5.00 log cfu ml<sup>-1</sup>), the growth curves of *Ent. cloacae* in Osmolite ( $p<0.001$ ) and Fortisip ( $p<0.001$ ) and Fortijuce ( $p<0.001$ ) were found to be significantly different from those of nutrient broth and Pulmocare. Numbers of *Ent. cloacae* did not increase in Fortijuce, but actually decreased to 4.35 log cfu ml<sup>-1</sup> at 24 hours. This was significantly less than in nutrient broth ( $p<0.001$ ). There were 2 hour lag phases in the growth of *Ent. cloacae* in nutrient broth, Osmolite and Fortisip but no detectable lag phase in Pulmocare. After the lag phase, growth in nutrient broth was constant (Table 3.2) with numbers increasing to 8.36 log cfu ml<sup>-1</sup> at 24 hours. In contrast, growth in the milk-based enteral nutrient solutions was in two phases. The first phase was a relatively fast growth rate and this lasted 12 hours in Osmolite and Fortisip and 20 hours in Pulmocare. At the end of this growth phase there were similar numbers in Osmolite and Fortisip (8.02 and 8.05 log cfu ml<sup>-1</sup> respectively), while there were slightly more in Pulmocare (8.37 log cfu ml<sup>-1</sup>). Growth rates during the second phase of growth were much slower than in the first phase. At the end of the experiment there were 8.82 log cfu ml<sup>-1</sup> in Osmolite, 9.02 log cfu ml<sup>-1</sup> in Fortisip and 8.74 log cfu ml<sup>-1</sup> in Pulmocare. Growth in Osmolite, Fortisip and Pulmocare was significantly more than in nutrient broth ( $p<0.001$ ,  $<0.001$  and  $<0.05$  respectively).

Growth from the lower inoculum was much more rapid than growth from the higher inoculum. At this inoculum size (approximately 2.00 log cfu ml<sup>-1</sup>), the growth curve of



**Figure 3.2:** Growth of *Enterobacter cloacae* in nutrient broth and enteral nutrient solutions. \*Each point represents the mean of three independent experiments with its SEM. ♦ Each point represents a mean of three plate counts with its SEM. \*Green: Nutrient broth, high inoculum; \*Magenta: Osmolite, high inoculum; \*Magenta: Fortisip, high inoculum; \*Cyan: Pulmocare, high inoculum; \*Red: Fortisip, high inoculum; ♦ Purple: Osmolite, low inoculum; ♦ Yellow: Nutrient broth, low inoculum; ♦ Purple: Osmolite, low inoculum.

**Table 3.2:** Mean Generation Times for *Enterobacter cloacae* growing in nutrient broth, Osmolite, Fortisip, Pulmocare and Fortijuce.

Inoculum Size	Growth Medium	Growth Phase 1	Growth Phase 2
5.00 log cfu ml <sup>-1</sup>	nutrient broth	130 min (2-24 hours)	N/A
	Osmolite	82 min (2-14 hours)	226 min (14-24 hours)
	Fortisip	80 min (2-14 hours)	186 min (14-24 hours)
	Pulmocare	115 min (0-20 hours)	195 min (20-24 hours)
	Fortijuce	N/G	
2.00 log cfu ml <sup>-1</sup>	nutrient broth	86 min (4-24 hours)	N/A
	Osmolite	62 min (4-24 hours)	N/A

N/G No growth of organism in medium.

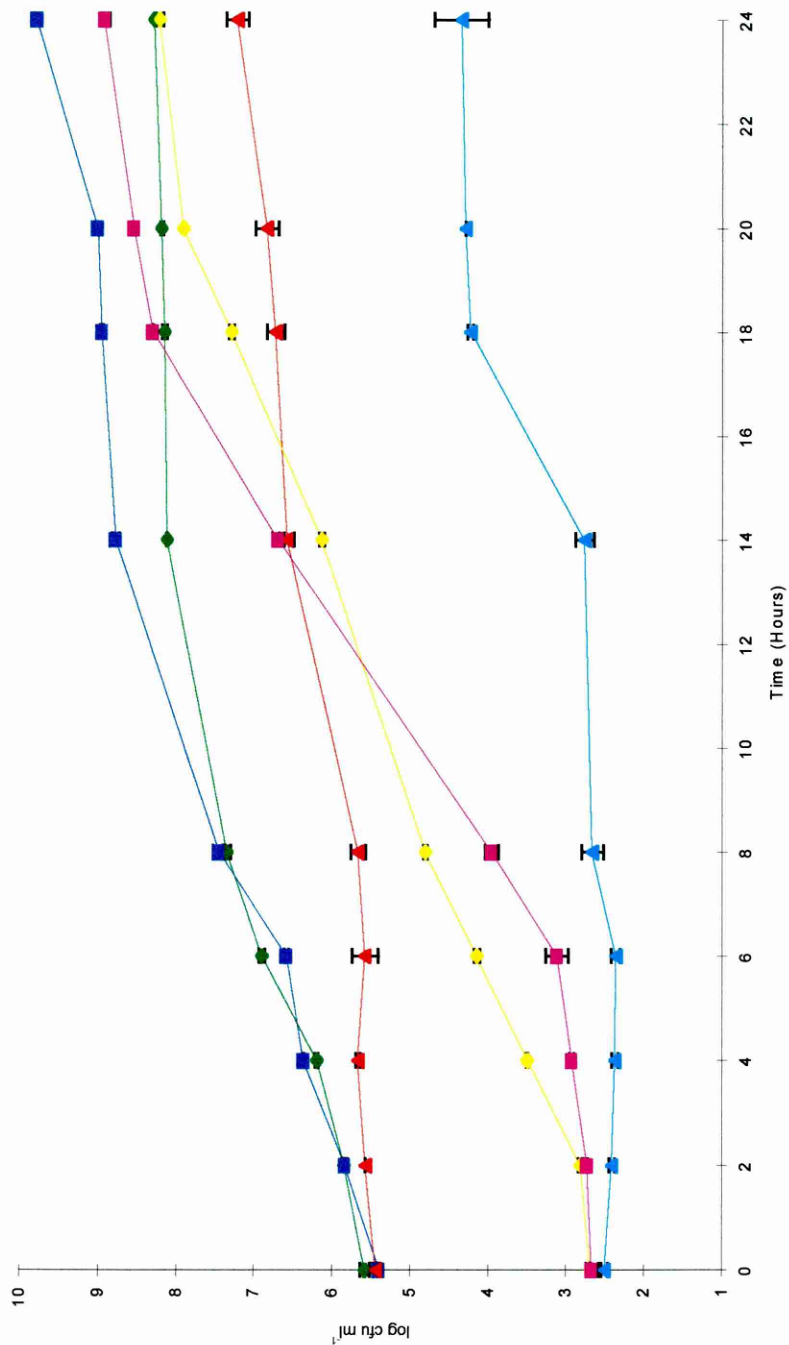
N/A Not applicable.

( ) Duration of growth phase.

*Ent. cloacae* in Osmolite was significantly different from that in nutrient broth ( $p<0.001$ ). There were 4 hour lag phases in nutrient broth and Osmolite. In nutrient broth, growth was similar to that of the higher inoculum with one phase of growth. The number in nutrient broth had increased to  $7.06 \log \text{ cfu ml}^{-1}$ . However, in contrast to growth at the higher inoculum there was only one phase of growth in Osmolite. The rate of growth was faster than that in nutrient broth and there were  $8.32 \log \text{ cfu ml}^{-1}$  *Ent cloacae* in Osmolite at the end of the experiment (Table 3.2). This level was significantly ( $p<0.001$ ) greater than in nutrient broth .

### **3.3.3 Growth of *K. aerogenes* in nutrient broth and enteral nutrient solutions**

Nutrient broth, Osmolite and Fortijuce were inoculated with two inoculum sizes of *K. aerogenes* (approximately  $5.00$  and  $2.00 \log \text{ cfu ml}^{-1}$ ). Viable counts were made over 24 hours (Figure 3.3). At the higher inoculum there were no lag phases in nutrient broth or Osmolite. However, there was a lag phase in Fortijuce which lasted 6 hours. Growth curves of *K. aerogenes* in Osmolite and Fortijuce were significantly different from that in nutrient broth ( $p<0.001$  and  $p<0.001$  respectively). *K. aerogenes* had two phases of growth in nutrient broth, a diauxic pattern of growth in Osmolite ( $p<0.001$ ) and slow intermittent growth in Fortijuce. The first phase of growth in nutrient broth consisted of relatively fast growth and lasted 14 hours, while growth in the second phase was much slower. MGT are shown in Table 3.3. The first period of growth in Osmolite was also the fastest and lasted 14 hours. This was followed by a 6 hour stationary phase in growth and a then a second phase of growth with a much slower rate. After the lag phase, *K. aerogenes* grew in Fortijuce for a further 18 hours at a rate slower than either nutrient broth or Osmolite. At the end of the experiment there were  $8.31 \log \text{ cfu ml}^{-1}$  in nutrient broth,  $9.80 \log \text{ cfu ml}^{-1}$  in Osmolite and  $7.22 \log \text{ cfu ml}^{-1}$  in Fortijuce. Numbers in Osmolite at 24 hour were significantly greater than in either nutrient broth ( $p<0.001$ ) or Fortijuce ( $p<0.001$ ).



**Figure 3.3:** Growth of *Klebsiella aerogenes* in nutrient broth and enteral nutrient solutions. Each point represents the mean of three independent experiments with its SEM. Green: Nutrient broth, high inoculum; Blue: Osmolite, high inoculum; Red: Fortijuice high inoculum; Yellow: Nutrient broth, low inoculum; Magenta: Osmolite, low inoculum; Cyan: Fortijuice low inoculum.

**Table 3.3:** Mean Generation Times for *Klebsiella aerogenes* growing in nutrient broth, Osmolite and Fortijuice.

Inoculum Size	Growth Medium	Growth Phase 1	Growth Phase 2
5.00 log cfu ml <sup>-1</sup>	nutrient broth	99 min (1-14 hours)	282 min (14-24 hours)
	Osmolite	75 min (0-14 hours)	93 min (20-24 hours)
	Fortijuice	186 min (8-24 hours)	N/A
4.00 log cfu ml <sup>-1</sup>	nutrient broth	91 (0-18 hours)	169 min (18-24 hours)
	Osmolite	81 min (0-18 hours)	
3.00 log cfu ml <sup>-1</sup>	nutrient broth	73 min (2-20 hours)	300 min (20-24 hours)
	Osmolite	66 min (2-18 hours)	148 min (18-24 hours)
2.00 log cfu ml <sup>-1</sup>	nutrient broth	64 min (2-20 hours)	233 min (20-24 hours)
	Osmolite	49 min (4-18 hours)	175 min (18-24 hours)
	Fortijuice	116 min (6-18 hours)	N/A

N/G No growth of organism in medium.

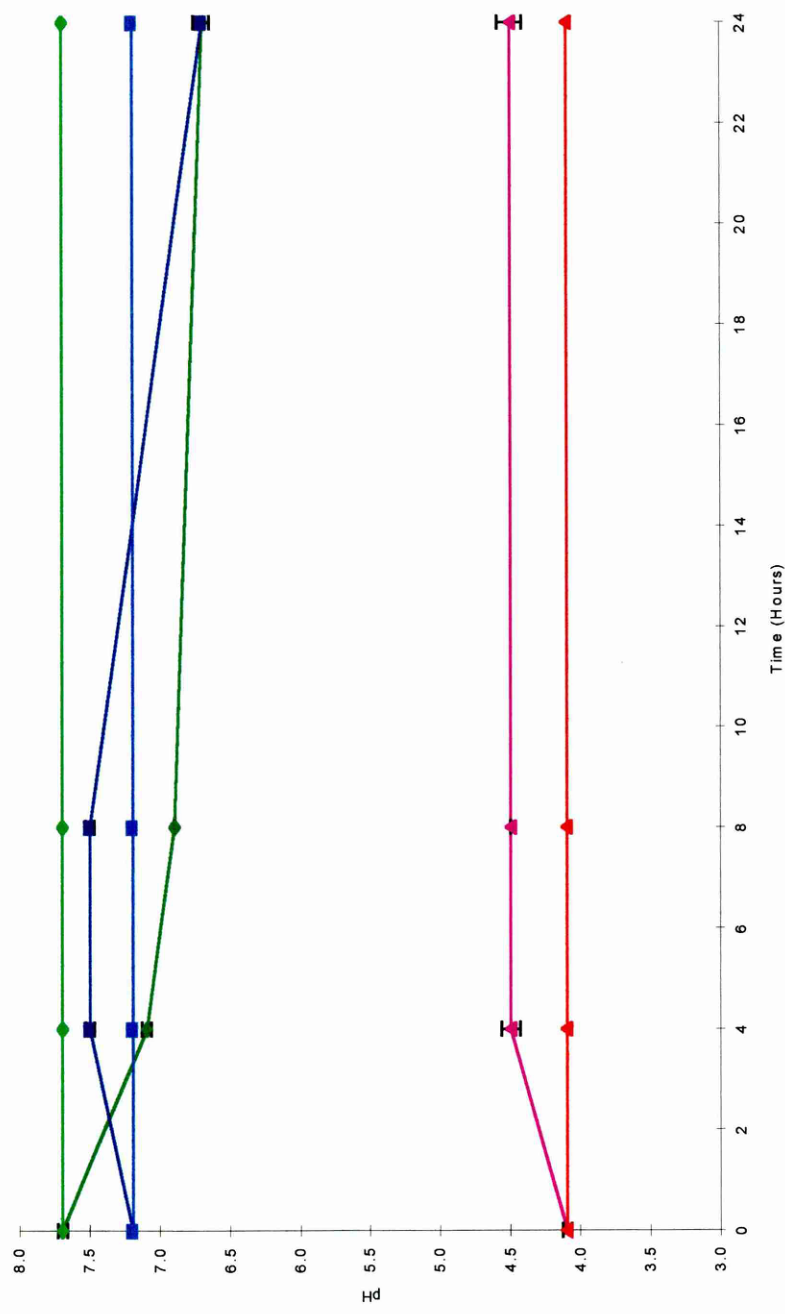
N/A Not applicable.

( ) Duration of growth phase.

When nutrient broth, Osmolite and Fortijuce were inoculated with approximately  $2.00 \log \text{ cfu ml}^{-1}$  there were 2, 4, and 14 hour lag phases respectively (Figure 3.3). *K. aerogenes* grew biphasically in both nutrient broth and Osmolite but the growth curves were statistically different when analysed using ANOVA ( $p < 0.001$ ). The first phases of growth were relatively fast and lasted 18 hours in nutrient broth and 14 hours in Osmolite. The second growth phases in these media were almost four times slower than initial growth rates and lasted until the end of the experiment. In contrast, there were 4 hours of exponential growth after the lag phase, followed by a stationary phase which lasted until the end of the experiment. During this brief exponential phase the Mean Generation Time was low and numbers of *K. aerogenes* increased from  $2.33 \log \text{ cfu ml}^{-1}$  to  $4.24 \log \text{ cfu ml}^{-1}$ . At the end of the experiment there were  $8.23 \log \text{ cfu ml}^{-1}$  in nutrient broth,  $8.93 \log \text{ cfu ml}^{-1}$  in Osmolite and  $4.35 \log \text{ cfu ml}^{-1}$  in Fortijuce. Numbers of *K. aerogenes* in nutrient broth at 24 hours were significantly greater in Osmolite than in nutrient broth ( $p < 0.001$ ).

### **3.3.4 pH changes in nutrient broth and enteral nutrient solutions during the growth of *K. aerogenes***

During the growth of *K. aerogenes* in nutrient broth, Osmolite and Fortijuce, measurements were made to investigate whether there were significant changes in the pH (Figure 3.4). There were statistically significant changes in pH in all the growth mediums at 4 hours. In nutrient broth, the pH decreased by 0.6 units over the first 4 hours and then by a further 0.4 units over the remainder of the experiment. The pH in Osmolite increased by 0.3 units for the first 8 hours but had decreased to 6.7 units by the end of the experiment. In contrast to the other growth media the pH of Fortijuce was relatively acidic (pH 4.1) at the beginning of the experiment. The pH in Fortijuce increased by 0.4 units over the first 4 hours and then was constant for the remainder of the experiment.



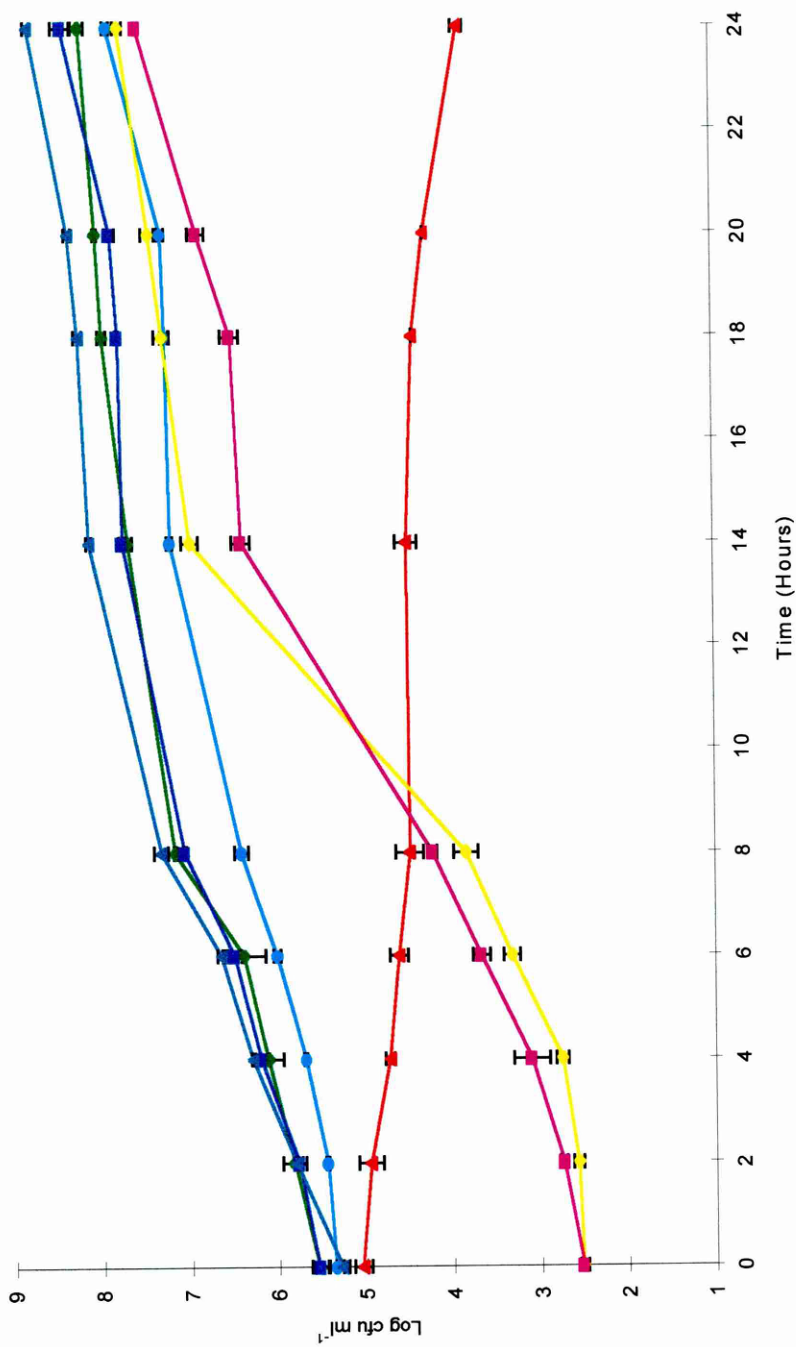
**Figure 3.4:** pH changes in the nutrient broth and enteral nutrient solutions during the growth of *Klebsiella aerogenes*. Each point represents the mean of three independent experiments with its SEM. ♦ Each point represents the pH at 0 hours. \*Dark green: Nutrient broth; ♦ Light green: Nutrient broth control; \*Dark blue: Osmolite; ♦ Mid-blue: Fortijuce; ♦ Magenta: Fortijuce control; \*Red: Fortijuce control.



### 3.3.5 Growth of *P. aeruginosa* in laboratory media and enteral nutrient solutions

At the high inoculum, the growth curves of *P. aeruginosa* in Fortisip and Fortijuce were significantly different from those in nutrient broth ( $p < 0.05$  and  $p < 0.001$  respectively), while there were no significant differences in the growth curves between nutrient broth and Osmolite or Pulmocare (Figure 3.5). There was a two hour lag phase in the growth of *P. aeruginosa* in nutrient broth and Osmolite. However, there were no lag phases in Fortisip or Pulmocare. Growth of *P. aeruginosa* occurred in two phases in nutrient broth but with a diauxic pattern in milk-based enteral nutrient solutions. Thus, growth in nutrient broth occurred at a relatively fast rate for 8 hours and then slowed considerably until the end of the experiment (Table 3.4). In contrast, growth occurred rapidly for 14 hours in Osmolite, Fortisip and Pulmocare but entered a stationary phase for 6 hours before resuming at a much slower rate. At the end of the experiment, numbers of *P. aeruginosa* had increased to  $8.20 \log \text{ cfu ml}^{-1}$  in nutrient broth,  $8.41 \log \text{ cfu ml}^{-1}$  in Osmolite,  $8.80 \log \text{ cfu ml}^{-1}$  in Fortisip and  $7.89 \log \text{ cfu ml}^{-1}$  in Pulmocare. Only the levels in Fortisip were significantly higher than those in nutrient broth ( $p < 0.01$ ). There was no growth of *P. aeruginosa* in Fortijuce, the fruit/water-based enteral nutrient solutions. Over the duration of the experiment, the numbers of *P. aeruginosa* declined to  $4.04 \log \text{ cfu ml}^{-1}$ . This concentration was significantly less than in nutrient broth ( $p < 0.001$ ).

When nutrient broth and Osmolite were inoculated with a low concentration of *P. aeruginosa* (approximately  $2.00 \log \text{ cfu ml}^{-1}$ ), the growth curves (Figure 3.5) of *P. aeruginosa* in Osmolite were significantly different ( $p < 0.001$ ) from that in nutrient broth. The growth curves were similar to those at the higher inoculum except that there was a 2 hour lag phase in nutrient broth, no lag phase in Osmolite and faster Mean Generation Times (Table 3.4). There were two phases of growth in nutrient broth and a diauxic pattern of growth in Osmolite. Thus, growth occurred in nutrient broth at a relatively fast rate for 12 hours and then the growth rate decreased dramatically.



**Figure 3.5:** Growth of *Pseudomonas aeruginosa* in nutrient broth and enteral nutrient solutions. \*Each point represents the mean of three independent experiments with its SEM. ♦ Each point represents a mean of three plate counts with its SEM. \*Green: Nutrient broth, high inoculum; \* Blue: Osmolite, high inoculum; \*Dark cyan: Fortisip, high inoculum; \*Cyan: Pulmocare, high inoculum; \*Red: Fortisip, high inoculum; ♦ Yellow: Nutrient broth, low inoculum; ♦ Magenta: Osmolite, low inoculum.

**Table 3.4:** Mean Generation Times for *Pseudomonas aeruginosa* growing in nutrient broth, Osmolite, Fortisip, Pulmocare and Fortijuce.

Inoculum Size	Growth Medium	Growth Phase 1	Growth Phase 2
5.00 log cfu ml <sup>-1</sup>	nutrient broth	81 min (2-8 hours)	278 min (8-24 hours)
	Osmolite	110 min (2-14 hours)	134 min (20-24 hours)
	Fortisip	89 min (0-14 hours)	150 min (20-24 hours)
	Pulmocare	136 min (0-14 hours)	120 min (20-24 hours)
	Fortijuce	N/G	
2.00 log cfu ml <sup>-1</sup>	nutrient broth	57 min (2-14 hours)	232 min (14-24 hours)
	Osmolite	65 min (0-14 hours)	138 min (18-24 hours)

N/G No growth of organism in medium.

N/A Not applicable.

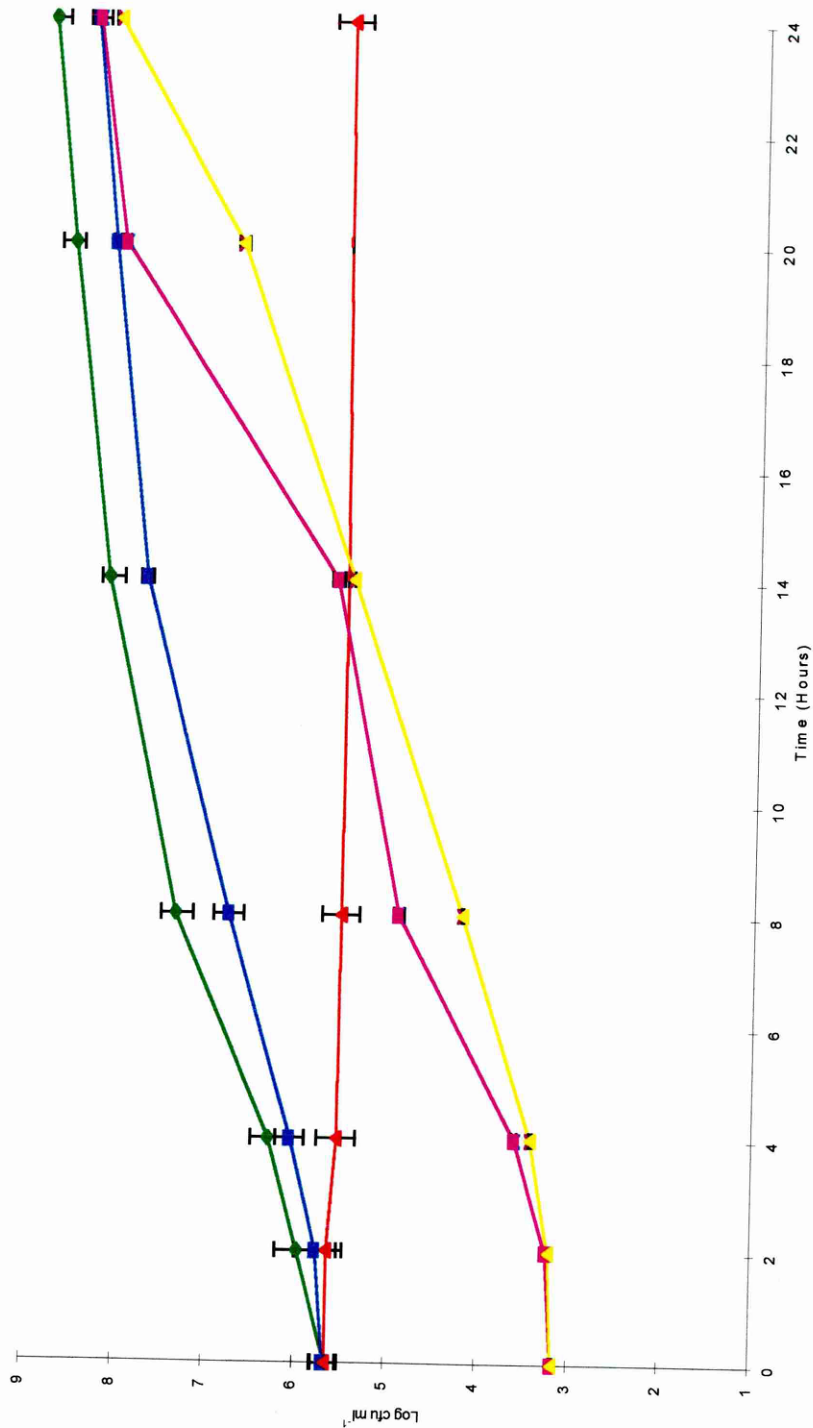
( ) Duration of growth phase.

However, growth in Osmolite occurred for 14 hours followed by a 4 hour stationary phase and a resumption of growth at a slower rate for the final 6 hours of the experiment. At the end of the experiment numbers had increased to 7.76 log cfu ml<sup>-1</sup> in nutrient broth and 7.55 log cfu ml<sup>-1</sup> in Osmolite. These levels were significantly different ( $p < 0.05$ ).

### **3.3.6 Growth of *S. aureus* in tryptone soya broth and enteral nutrient solutions**

Tryptone soya broth, Osmolite and Fortijuce were inoculated with approximately 5.00 log cfu ml<sup>-1</sup> of *S. aureus*, and viable counts were made over 24 hours (Figure 3.6). The growth curve of *S. aureus* in Osmolite was not significantly different from that in tryptone soya broth but the growth curve from Fortijuce was significantly different ( $p < 0.001$ ). There was a two hour lag phase in tryptone soya broth and a four hour lag phase in Osmolite. The first phase of growth in both tryptone soya broth and Osmolite had a relatively short Mean Generation Time and lasted 12 and 10 hours respectively (Table 3.5). After this time, growth slowed considerably and continued at this rate until the end of the experiment, by which time numbers had increased to 8.84 log cfu ml<sup>-1</sup> in tryptone soya broth and 8.38 log cfu ml<sup>-1</sup> in Osmolite. In contrast to tryptone soya broth and Osmolite there was no significant increase in numbers of *S. aureus* in Fortijuce over 24 hours.

Tryptone soya broth and Osmolite were also inoculated with a lower number of *S. aureus*, (approximately 3.00 log cfu ml<sup>-1</sup>). With this lower inoculum, the growth curve of *S. aureus* in tryptone soya broth was found to be significantly different from that in Osmolite ( $p < 0.001$ ). There was a two hour lag phase in Osmolite but no lag phase in tryptone soya broth. Growth occurred in two phases in tryptone soya broth. The first phase of growth in tryptone soya broth was a relatively fast rate and lasted until the 20 hour time interval (Table 3.5), while the second phase of growth had a much slower growth rate and lasted from 20 hours to the end of the experiment. In contrast, the



**Figure 3.6:** Growth of *Staphylococcus aureus* in tryptone soya broth and enteral nutrient solutions. \*Each point represents the mean of three independent experiments with its SEM. ♦ Each point represents a mean of three plate counts with its SEM. \* Green: Tryptone soya broth, high inoculum; \* Blue: Osmolite, high inoculum; \* Red: Fortijuce, high inoculum; ♦ Yellow: Tryptone soya broth, low inoculum; ♦ Magenta: Osmolite, low inoculum.

**Table 3.5:** Mean Generation Times for *Staphylococcus aureus* growing in tryptone soya broth and enteral nutrient solutions.

Inoculum Size	Growth Medium	Growth Phase 1	Growth Phase 2
5.00 log cfu ml <sup>-1</sup>	tryptone soya broth	100 min (2-14 hour)	262 min (14-24 hour)
	Osmolite	108 min (4-14 hour)	282 min (14-24 hour)
	Fortijuce	N/G	
3.00 log cfu ml <sup>-1</sup>	tryptone soya broth	52 min (0-20 hour)	200 min (20-24 hour)
	Osmolite	81 min (2-24 hour)	N/A

N/G No growth of organism in medium.

N/A Not applicable.

( ) Duration of growth phase.

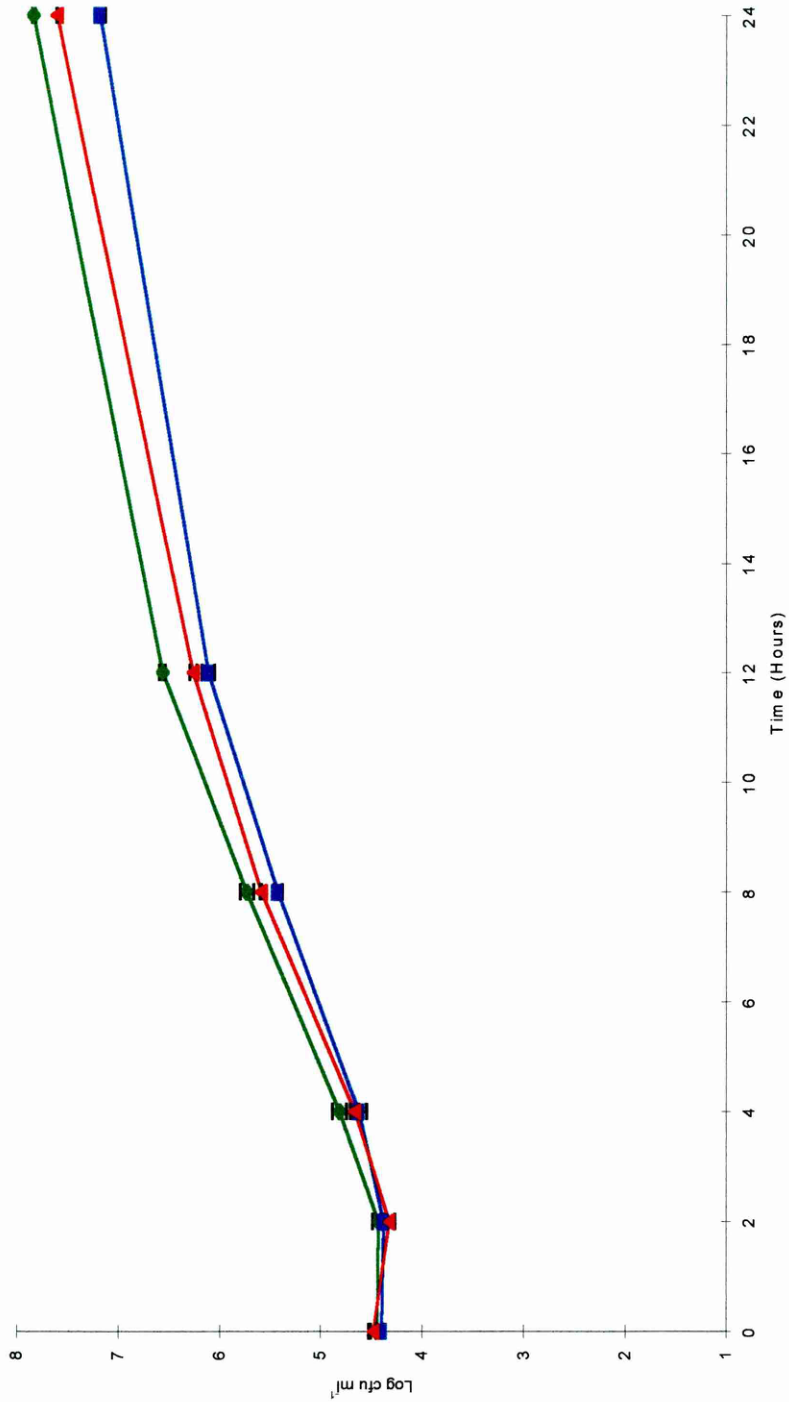
growth rate in Osmolite was constant over the period of the experiment. At the end of the experiment, numbers had increased to 8.39 log cfu ml<sup>-1</sup> in tryptone soya broth and 8.13 log cfu ml<sup>-1</sup> in Osmolite. These concentrations of *S. aureus* were not found to be significantly different.

### **3.3.7 Growth of *C. albicans* in Sabouraud broth and enteral nutrient solutions**

Sabouraud broth, Osmolite and Fortijuce were inoculated with approximately 4.00 log cfu ml<sup>-1</sup> and viable counts were made over 24 hours (Figure 3.7). Although there were two phases of exponential growth in all media, the growth curve of *C. albicans* in Osmolite was found to be significantly different ( $p < 0.001$ ) from either *C. albicans* in Sabouraud broth or Fortijuce. In addition, there were a 4 hour lag phase in Osmolite, while there was only two hour lag phases in either Sabouraud broth or Fortijuce. The first exponential growth phases had relatively low Mean Generation Times (Table 5.6) and lasted 8 hours (Osmolite) or 10 hours (Sabouraud broth and Fortijuce). Numbers increased to 6.57 log cfu ml<sup>-1</sup> in Sabouraud broth, 6.12 log cfu ml<sup>-1</sup> in Osmolite and 6.26 log cfu ml<sup>-1</sup> in Fortijuce. This was followed by a phase of slower growth which lasted until the end of the experiment. At 24 hours numbers had risen to 7.84 log cfu ml<sup>-1</sup> in Sabouraud broth, 7.18 log cfu ml<sup>-1</sup> in Osmolite and 7.61 log cfu ml<sup>-1</sup> in Fortijuce. Numbers in Osmolite at this point were found to be significantly less than in Sabouraud broth ( $p < 0.001$ ) and in Fortijuce ( $p < 0.001$ ).

### **3.3.8 The effect of inoculum size on the growth kinetics of *K. aerogenes* in nutrient broth and Osmolite**

Nutrient broth and Osmolite, a typical milk-based enteral nutrient solution, were inoculated with four inocula sizes of *K. aerogenes* (approximately 5.00, 4.00, 3.00 and 2.00 log cfu ml<sup>-1</sup>). Viable counts were made over 24 hours (Figure 3.8) and MGT were calculated (Table 3.3). Growth in nutrient broth or Osmolite from 4.00, 3.00 and 2.00 log



**Figure 3.7:** Growth of *Candida albicans* in Sabouraud broth and enteral nutrient solutions. Each point represents the mean of three independent experiments with its SEM. Green: Sabouraud broth, high inoculum; Blue: Osmolite, high inoculum; Red: Fortijuce, high inoculum.



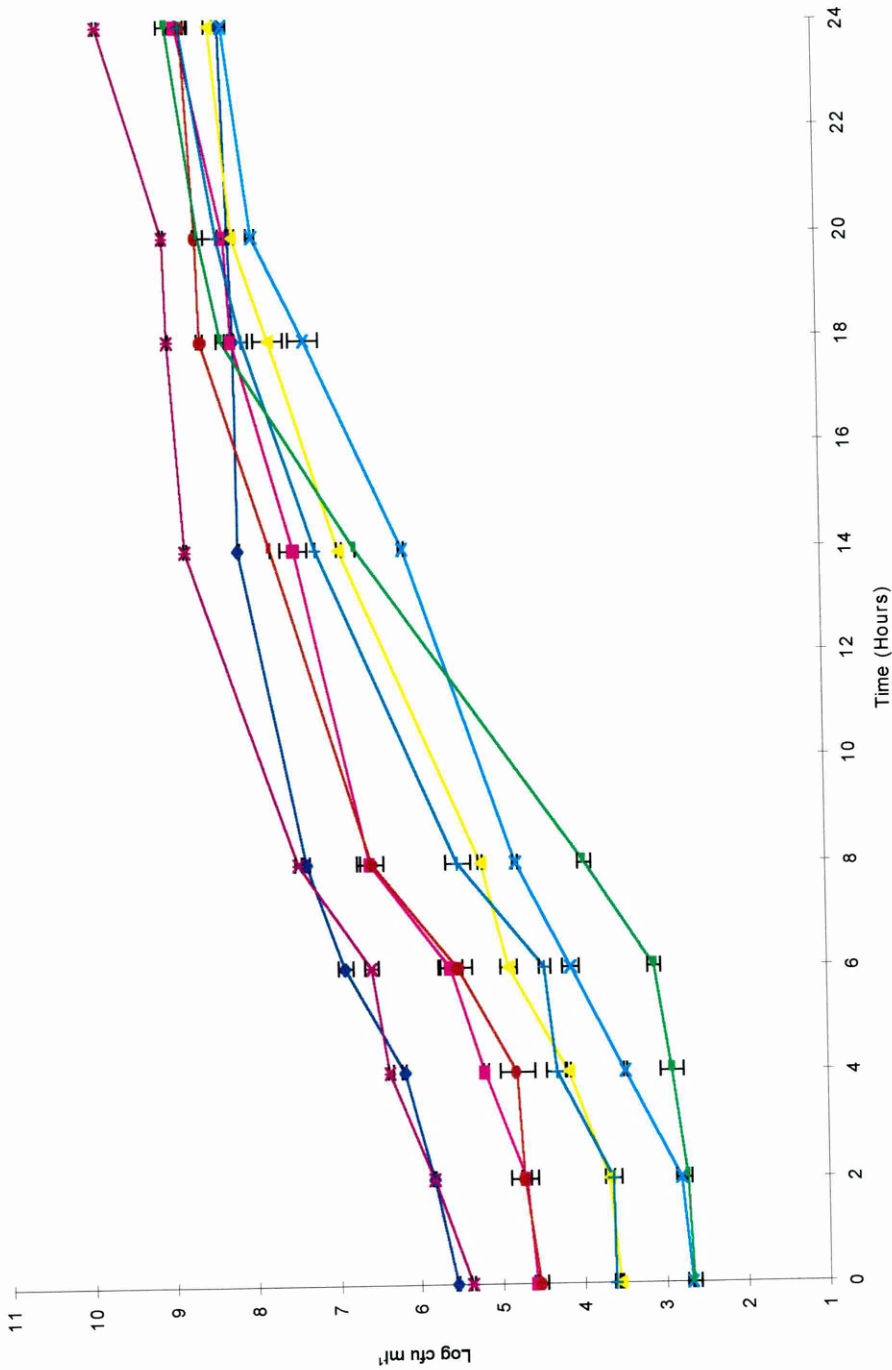
**Table 3.6:** Mean Generation Times for *Candida albicans* growing in Sabouraud broth, Osmolite and Fortijuice.

Inoculum Size	Growth Medium	Growth Phase 1	Growth Phase 2
5.00 log cfu ml <sup>-1</sup>	Sabouraud broth	85 min (2-12 hours)	171 min (12-24 hours)
	Osmolite	97 min (4-12 hours)	204 min (12-24 hours)
	Fortijuice	94 min (2-12 hours)	161 min (12-24 hours)

N/G No growth of organism in medium.

N/A Not applicable.

( ) Duration of growth phase.



**Figure 3.8:** The effect of inoculum size on the growth of *K. aerogenes* in nutrient broth and Osmolite. Each point represents a mean three independent experiments with its SEM. Blue: Nutrient broth, 5.00 log cfu ml<sup>-1</sup> inoculum; Magenta: nutrient broth 4.00 log cfu ml<sup>-1</sup> inoculum; Yellow: Nutrient broth, 3.00 log cfu ml<sup>-1</sup> inoculum; Cyan: Nutrient broth, 2.00 log cfu ml<sup>-1</sup> inoculum; Purple: Osmolite, 5.00 log cfu ml<sup>-1</sup> inoculum; Red: Osmolite, 4.00 log cfu ml<sup>-1</sup> inoculum; Dark Cyan: Osmolite, 3.00 log cfu ml<sup>-1</sup> inoculum; Green: Osmolite, 2.00 log cfu ml<sup>-1</sup> inoculum.

cfu ml<sup>-1</sup> inocula were compared with the growth from the highest inoculum (5.00 log cfu ml<sup>-1</sup>) using Students t-tests (Table 3.7).

*K. aerogenes* grew rapidly in nutrient broth from all inoculum sizes. In tubes where either a 5.00 or 4.00 log cfu ml<sup>-1</sup> inoculum was used, there were no lag phases in bacterial growth. However, in tubes where the starting inoculum was 3.00 log cfu ml<sup>-1</sup> there was a 2 hour lag phase and in tubes where a 2.00 log cfu ml<sup>-1</sup> inoculum was used, a 4 hour lag phase was measured. Growth from all sizes of starting inoculum occurred in two exponential phases. Mean Generation Times during the first phase of growth increased with the size of starting inoculum. For example, in tubes inoculated with 5.00 log cfu ml<sup>-1</sup> the Mean Generation Time in the first growth phase was 91 minutes, while in tubes inoculated with 2.00 log cfu ml<sup>-1</sup> the Mean Generation Time was only 64 minutes. There was no such pattern in the Mean Generation Times calculated for the second exponential growth phases.

Significant differences ( $p < 0.05$ ) in amount of growth in tubes inoculated with 2.00, 3.00 and 4.00 log cfu ml<sup>-1</sup> *K. aerogenes* compared to the amount of growth in the tubes inoculated with 5.00 log cfu ml<sup>-1</sup> were observed until 18 hours. At this time point, neither of the amounts of growth in tubes inoculated with 4.00 or 3.00 log cfu ml<sup>-1</sup> were significantly different from the amounts of growth in tubes inoculated with 5.00 log cfu ml<sup>-1</sup> (Data not available for the tube inoculated with 2.00 log cfu ml<sup>-1</sup>). Similarly, at 24 hours the amounts of growth in tubes inoculated with 2.00 and 3.00 log cfu ml<sup>-1</sup> were not significantly different from the amounts of growth in tubes inoculated with 5.00 log cfu ml<sup>-1</sup>. However, the amount of growth in tubes inoculated with 4.00 log cfu ml<sup>-1</sup> was significantly different ( $p < 0.05$ ) to the amount of growth in tubes inoculated with 5.00 log cfu ml<sup>-1</sup> at 24 hours. Concentrations of *K. aerogenes* in tubes at the end of the experiment were in the range 8.82 to 8.23 log cfu ml<sup>-1</sup>.

**Table 3.7:** Comparison of growth in tubes inoculated with different concentrations of *K. aerogenes*.

Growth Medium	Inoculum Size log cfu ml <sup>-1</sup>	4 hours log cfu ml <sup>-1</sup>	8 hours log cfu ml <sup>-1</sup>	14 hours log cfu ml <sup>-1</sup>	18 hours log cfu ml <sup>-1</sup>	20 hours log cfu ml <sup>-1</sup>	24 hours log cfu ml <sup>-1</sup>
Nutrient Broth	5.00	6.20	7.36	8.13	8.16	NR	8.31
	4.00	5.21 p<0.001	6.59 p<0.05	7.46 p<0.05	8.18 NS	NR	8.82 p<0.05
	3.00	4.20 p<0.001	5.24 p<0.001	6.9 p<0.001	7.73 NS	NR	8.40 NS
	2.00	3.50 p<0.001	4.81 p<0.001	6.14 p<0.001	NR	7.92	8.23 NS
Osmolite	5.00	6.37	7.46	NR	8.97	9.02	9.80
	4.00	4.82 p<0.05	6.56 p<0.01	NR	8.56 p<0.05	8.61 p<0.001	8.76 p<0.001
	3.00	4.34	5.51 p<0.005	NR	8.05 p<0.005	8.35 p<0.05	8.78 p<0.005
	2.00	3.12	4.25 p<0.001	NR	8.31 p<0.001	8.56 p<0.005	8.93 p<0.005

p- values are the significance of results in comparison to growth in tubes inoculated with 5.00 log cfu ml<sup>-1</sup> *K. aerogenes*. NR: No measurement taken at this time. NS: No statistical difference between result and amount of growth in tubes inoculated with 5.00 log cfu ml<sup>-1</sup>.

*K. aerogenes* also grew rapidly in Osmolite from all inoculum sizes. In tubes inoculated with a 5.00 log cfu ml<sup>-1</sup> concentration of *K. aerogenes* there was no lag phase. However, in tubes inoculated with a 4.00 log cfu ml<sup>-1</sup> concentration of *K. aerogenes* there was a 4 hour lag phase and in tubes inoculated with 2.00 or 3.00 log cfu ml<sup>-1</sup> there were 2 hour lag phases. There was one phase of exponential growth in tubes inoculated with either 5.00 or 2.00 log cfu ml<sup>-1</sup> *K. aerogenes*. Whereas in the tubes inoculated with 4.00 or 3.00 log cfu ml<sup>-1</sup> *K. aerogenes* there were two exponential growth phases. With the exception of the result for tubes inoculated with 4.00 log cfu ml<sup>-1</sup>, mean generation times in this first exponential growth phase increased with starting inoculum size. For example, in tubes inoculated with 5.00 log cfu ml<sup>-1</sup>, the Mean Generational Time was 75 minutes, while in tubes inoculated with 2.00 log cfu ml<sup>-1</sup> the Mean Generation Time was 49 minutes. There was no such pattern to the Mean Generation Times from the second exponential growth phase in tubes inoculated with either 4.00 log cfu ml<sup>-1</sup> or 3.00 or cfu ml<sup>-1</sup>.

Significant differences were observed in the amount of growth in tubes inoculated with 4.00, 3.00 or 2.00 log cfu ml<sup>-1</sup> *K. aerogenes* compared to tubes inoculated with 5.00 log cfu ml<sup>-1</sup>, throughout the course of the experiment. Concentrations of *K. aerogenes* at the end of the experiments were in the range 9.80 to 8.76 log cfu ml ml<sup>-1</sup>.

### 3.4 DISCUSSION

The parameters set in these experiments, such as incubation temperature and incubation length were selected to reflect normal clinical conditions present during enteral feeding of patients. Growth measurements were made over 24 hours to simulate the maximum hanging time for safe enteral feeding as recommended by the Microbiological Guidelines for Administration of Enteral Feeds (Anderton *et al*, 1986). Although microorganisms may interact with enteral nutrient solutions at temperatures which range from room temperature to that of the body, room temperature (25°C) was selected to minimize the number of variables in the study. Microbial strains used in this study have all been shown to be strains which commonly contaminate enteral feeding systems and have been used in other similar growth studies (Anderton, 1985; Oversen *et al*, 1991). The laboratory media selected for each microbe were the most commonly used media for the culture of the organisms concerned. Osmolite and Fortijuce were selected for study as they were considered representative of their type of enteral nutrient solutions (milk-based and "fruit-based" respectively). Fortisip and Pulmocare were included in the growth studies of *E. coli*, *Ent. cloacae* and *P. aeruginosa* because they were to be included in pathogenicity studies of these organisms. In addition, these enteral nutrient solutions had slightly different compositions and physical properties which were of interest.

The first aim of this chapter was to measure the kinetic parameters of microbial growth in enteral nutrient solutions. Bacteria grew rapidly in laboratory media (nutrient broth or tryptone soya broth) increasing to numbers in the range 8.21 to 9.00 log cfu ml<sup>-1</sup> after 24 hours at 25°C, while numbers of yeast increased to 7.84 log cfu ml<sup>-1</sup> (Sabouraud broth). Microbial growth in milk-based enteral nutrient solutions was rapid but in most cases statistically greater than in laboratory media. In this type of enteral feed, bacterial numbers increased to levels of 8.38 to 9.20 log cfu ml<sup>-1</sup> after 24 hours. These results were similar to the findings of Oversen *et al* (1991) who found that a range of Gram-positive and Gram-negative bacteria grew well in Osmolite, increasing from 10<sup>3</sup>-10<sup>5</sup> to 10<sup>7</sup>-10<sup>8</sup> cfu ml<sup>-1</sup> after 24 hours at 25°C. However, in contrast to Oversen *et al* (1991)

this study found that *C. albicans* also grew in Osmolite, with numbers increasing to 7.84 log fu ml<sup>-1</sup> after 24 hours culture at 25°C in Osmolite. This difference in results may have been because Oversen and co-workers (1991) used a different strain of *C. albicans*.

In general, growth of microbes occurred in two phases but *P. aeruginosa* had a diauxic pattern of growth in milk-based enteral nutrient solutions at the high inoculum. Growth occurred for 14 hours at a relatively fast rate (MGT=110 to 136 minutes), followed by a 6 hour stationary phase and a resumption of growth at a slightly slower rate (MGT=120 to 150 minutes). This pattern of growth was not observed in laboratory media, indicating that *P. aeruginosa*, at high cell concentrations, may produce enzymes in a diauxic stationary phase to enable it to utilize the more complex nutrients present in milk-based enteral nutrient solutions. These include dextrans, lipids and semi-hydrolysed proteins.

When milk-based enteral nutrient solutions were inoculated with the higher inoculum size of other microorganisms, the first phase of growth usually lasted approximately 14 to 18 hours. This first phase has a relatively fast growth rate and was followed by a second phase of growth with a much slower rate, lasting a further 6 to 10 hours. Milk-based enteral nutrient solutions inoculated with the lower inoculum size had either extended first exponential phases lasting up to 20 hours with fast growth rates and short second exponential phases lasting 4 hours or had only one exponential growth phase. The slowing of growth in milk-based enteral nutrient solutions generally occurred as numbers rose above 7.5 log cfu ml<sup>-1</sup>. This may indicate an exhaustion of nutrients or a toxic build-up of microbial waste products (Stanier *et al*, 1977).

In contrast to milk-based enteral nutrient solutions, Fortijuce only supported the growth of *C. albicans* and *K. aerogenes* and was bacteristatic to *E. coli*, *Ent. cloacae*, *P. aeruginosa* and *S. aureus*. This result was not surprising because Fortijuce has a low pH and a high osmolarity (Table 3.8). It is well known that high concentrations of

**Table 3.8:** Major ingredients contained in enteral nutrient solutions and physical properties. Data taken from product packaging. Ross Products Division, Abbott Laboratories Limited, Maidenhead, Berkshire, UK and Nutricia Clinical Care Ltd, Trowbridge, Wiltshire UK.

Fortijuce	Fortisip	Osmolite	Pulmocare.
Fruit-based	Milk-based	Milk-based	Milk-based
Water Maltodextrin Sucrose Whey protein hydrosylate Gelatin hydrosylate Casein hydrosylate  Citric acid Flavourings	Water Maltodextrin Sucrose Whey protein hydrosylate Gelatin hydrosylate Casein hydrosylate  Flavourings	Water maltodextrin Caseinates sunflower oil  canola oil Fractionated coconut oil soy protein isolate Emulsifyer E322	Water Corn oil Caseinates Sucrose  Maltodextrin Emulsifyer: E322
pH *	pH*	pH*	pH*
4.1	6.9	6.8	6.9
Osmolarity	Osmolarity	Osmolarity	Osmolarity
670 mOsmL <sup>-1</sup>	325 mOsmL <sup>-1</sup>	342 mOsmL <sup>-1</sup>	505 mOsmL <sup>-1</sup>

\*Mean of pH measurements taken from 3 separate cans or tetra-brik packs using method described in Section 3.2.2.2. pH measurement were not supplied by manufacturer.



hydrogen ions can inhibit the uptake of nutrients, alter the structure molecules such as DNA and ATP and inhibit the activity of enzymes (Jay, 1992). In addition, the high osmolarity of this product will increase osmotic stress on cells extending the lag phase indefinitely. It was expected that *C. albicans* would grow in Fortijuce because Sabouraud broth, the standard laboratory media for this organism, also has a low pH and a high sugar content. In fact, there was no significant difference between the numbers of *C. albicans* after 24 hours of growth at 25°C in Fortijuce and laboratory media. More interesting was the growth of *K. aerogenes* in the Fortijuce. Although the amount of growth was significantly less ( $p < 0.001$ ) than in nutrient broth, numbers of *K. aerogenes* increased by  $1.78 \log \text{ cfu ml}^{-1}$  at the higher inoculum and  $1.84 \log \text{ cfu ml}^{-1}$  at the lower inoculum. The reason for the greater tolerance of *K. aerogenes* to the conditions in Fortijuce compared with other Gram-negative bacteria is unclear. It may be due to the protection conferred to this organism by its large capsule. Alternatively, some microorganisms are known to alter the pH of their environment, enabling them to grow. For example, *Ent. aerogenes* produces acetoin from pyruvic acid to increase the pH of its environment when growing in acidic conditions (Jay, 1992). To examine whether *K. aerogenes* increased the pH of its environment in a similar manner, the pH was monitored during growth in Fortijuce. The results indicated that there was a small but statistically significant ( $p < 0.05$ ) increase in the pH during the experiment. However, the increase was too small to distinguish it from the in-built error of the pH measuring equipment.

The second aim of this chapter was to investigate those factors which affect growth in enteral nutrient solutions and are pertinent to improving enteral feeding practice. The main strategy employed by clinicians and manufacturers to prevent contamination of enteral nutrient solutions has been to develop enteral feeding systems and protocols which minimize the amount of microorganisms entering the feeding system. The efficacy of "closed" systems at preventing contamination has been discussed previously in Section 1.5.1.2. The evidence in the literature suggests that in "closed" systems the amount of

contamination is reduced but the presence of contaminating microorganisms is not eliminated. Despite this evidence, authors including Wagner *et al* (1994), Dentinger *et al* (1995) and Weenk *et al* (1995) have suggested that it may be possible to increase the volume of enteral feeding reservoirs and increase the hang time when using "closed" enteral feeding systems. This chapter investigated the safety of these recommendations by studying the effect of inoculum size on growth of *K. aerogenes* in enteral nutrient solutions and laboratory media. The results of this investigation have shown that growth from low inoculum sizes is more rapid than growth from higher inocula. At the end of the experiment (24 hours), which represented the maximum hang time for most "closed" enteral feeding systems, numbers of bacterial were very high ( $8.93 \log \text{ cfu ml}^{-1}$ ), even when a low inoculum (approx.  $2.00 \log \text{ cfu ml}^{-1}$ ) was used. These results are not entirely unexpected because at lower bacterial cell concentrations there is less competition for nutrients and so growth rates tend to be higher. However, the importance of these results for clinical practice is clear: either hang-times should be reduced (See Chapter 6) or an alternative to using "closed" enteral feeding systems as a method of preventing contamination should be found.

Oversen and co-workers (1991) suggested that decreasing pH and increasing osmolarity may be a means of inhibiting the growth of microorganisms in a wide range of enteral nutrient solutions. Unfortunately, this is difficult to test in enteral nutrient solutions because it is impossible to control all the intrinsic factors which affect microbial growth. However, the results of this study did show that the low pH and high osmolarity of Fortijuce completely inhibited the growth of most microorganisms tested, with the exception of *K. aerogenes* and *C. albicans* which were still able to grow. Similarly, the amount of growth of *E. coli* and *P. aeruginosa* in Pulmocare (which has a high osmolarity) was significantly less ( $p < 0.05$ ) than in other milk-based enteral feeds after 24 hours, while there was no significant difference between numbers of *Ent. cloacae* in any of the milk-based enteral nutrient solutions after 24 hours. The effect of the high osmolarity on microbial growth in Pulmocare was difficult to assess because of

compositional differences between Pulmocare and other milk-based feeds (Table 3.9) but in any case it was too small to have any substantial effect on the numbers of microbes in the enteral nutrient solutions at the end of the experiment.

In addition to the microbiological inadequacies of using low pH and high osmolality to prevent microbial growth in enteral nutrient solutions, there are also clinical reasons which could make products with these properties detrimental to the patient. Decreasing the pH in milk-based enteral nutrient solutions would cause the precipitation of caseinates present in this type of feed (Table 3.8) and may also counteract H<sub>2</sub> antagonists and antacids given concomitantly with many nasogastric feeds. Similarly, increased osmolality is known to lower patient tolerance of enteral nutrient solutions (Burns and Jairath, 1994) and is often cited as a reason for diluting enteral formulae, a practice which is known to cause microbial contamination of the feeding system (Anderton, 1993).

**Table 3.9:** Enteral nutrient solutions compositional data used in growth studies (per 100 ml).

	<b>Fortijuce</b>	<b>Fortisip</b>	<b>Osmolite</b>	<b>Pulmocare.</b>
Type	Fruit-based	Milk-based	Milk-based	Milk-based
Feeding route	Sip	Sip	Tube	Tube
Application	General	General	General	Pulmocare
Manufacturer	Nutricia	Nutricia	Ross-Abbott	Ross-Abbott
Energy	523 kJ	635 kJ	424 kJ	624 kJ
Fat	0 g	6.5 g	4 g	9.2 g
Carbohydrate sugars	9.69 g	1.43 g	0.7 g	0
Carbohydrate	18.3 g	16.1 g	13.6 g	10.56 g
protein	4 g	5.0 g	3.4 g	6.25 g
sodium	12 mg	3.5 mmol	0.09 g	5.65 mmol
potassium	26 mg	3.8 mmol	3.79mmol	4.46 mmol
chloride	88 mg	2.25 mmol	3.84 mmol	4.75 mmol
calcium	80 mg	1.25 mmol	72 mg	106 mg
phosphorus	60 mg	50 mg	72 mg	106 mg
magnesium	30 mg	22 mg	29 mg	42 mg
iron	1.5 mg	1.5 mg	1.35 mg	2.1 mg
zinc	1.5 mg	1.05 mg	1.8 mg	2.8 mg
copper	0.23 mg	0.6 mg	0.17 mg	0.26 mg
manganese	0.45 mg	0.15 mg	0.38 mg	0.6 mg
iodide	15.4 µg	9 µg	11.0 µg	16 µg
selenium	7.4 µg	0	6.5 µg	10 µg
chromium	5 µg	0	6.5 µg	10 µg
molybdenum	20.6 µg	0	12.0 µg	18 µg
Vitamin A	80 µg	105 µg	108 µg	158 µg
Vitamin D	1.02 µg	0.75 µg	0.73 µg	1.05 µg
Vitamin E	1.7 mg	4.8 mg	3.2 mg	4.8 µg
Vitamin K	6.1 mg	0	5.2 µg	8.4 µg
Vitamin C	0	7.5 mg	13.2 mg	32.0 mg
thiamin	0.15 mg	0.11 mg	0.16 mg	0.32 mg
riboflavin	0.17 mg	0.15 mg	0.18 mg	0.36 mg
Vitamin B6	0	0.15 mg	0.22 mg	0.42 mg
Vitamin B2	0.2 mg	0	0	0
Vitamin B12	0.17 µg	0.3 µg	0.68 µg	1.3 µg
inositol	34.6 mg	34.5 mg	0	0
niacin	1.85 mg	1.5 mg	0	4.2 mg
folic acid	20 µg	37.5 µg	44 µg	84 mg
biotin	30 µg	22.5 µg	33µg	64 mg
pantothenic acid	0.64 mg	0.75 mg	1.1 mg	2.1 mg
choline	66.7 mg	67.5 mg	56.0 mg	64 mg
water	80 g	78.4 g	85 g	78.6 g

Data supplied by Abbott Laboratories Limited, Maidenhead, Berkshire, UK and Nutricia Clinical Care Ltd, Trowbridge, Wiltshire UK

### 3.5 SUMMARY

Growth of microorganisms in milk-based enteral nutrient solutions was rapid and in most cases similar to standard laboratory media used in general culture of these microbes. In contrast, the growth of bacteria, with the exception of *K. aerogenes*, was completely inhibited in "fruit"-based enteral nutrient solutions, while the yeast (*C. albicans*) grew rapidly in this type of enteral feed. Results showed increased mean generation times when the initial inoculum size of microorganisms was reduced and demonstrated the need for careful reconsideration of the hang-times for "closed" enteral feeding systems. Although in many cases, microbial growth was significantly less in enteral nutrient solutions which had high osmolarity or a combination of low pH and high osmolarity, these factors were either insufficient to inhibit a broad-spectrum of microorganisms or did not prevent substantial growth in enteral nutrient solution. Finally, the growth studies in this chapter reiterated the need to develop alternative strategies for maintaining the microbiological quality of enteral nutrient solutions and provided the necessary information for microbial pathogenicity studies in enteral nutrient solutions.

## CHAPTER 4

# INHIBITION OF MICROORGANISMS BY OIL OF FENNEL, PARABENS AND COMBINATIONS OF OIL OF FENNEL WITH PARABENS

### 4.1 INTRODUCTION

Oil of fennel is the essential oil from the seeds and foliage of the fennel plant (*Foeniculum vulgare*). Its main components are *trans*-anethole (52-86%), fenchone (1-14%) and estragole (2-7%) pinene, camphene, (+)- $\alpha$ -phellandrene, dipentene, p-hydroxyphenylacetone, anisaldehyde, anisic acid, and 1,3-dimethylbutadiene. It is similar in composition to the oils of aniseed, star anise and basil (Max, 1992; Tisserand and Balacs, 1995).

There has been very little research into the antimicrobial properties of oil of fennel. Deans and Richie (1987) examined the activity of oil of fennel at four different concentrations against 25 genera of bacteria using the agar well diffusion method. They found that oil of fennel had activity against 6 species at concentrations of 100% and 50%, while at concentrations of 20% and 10% it was inhibitory to 11 species. *Aeromonas hydrophila* and *Moraxella* sp. were the only genera inhibited at all concentrations, while *Leuconostoc cremoris*, *Enterobacter aerogenes* and *Proteus vulgaris* were inhibited at three concentrations of oil of fennel (Deans and Richie, 1987). These workers found that two food pathogens, *Salmonella* spp. and *Bacillus cereus* were inhibited at 20% and 10% . These findings suggest there was not a linear relationship between the concentration of oil of fennel and its antimicrobial activity against the species tested or that the ethanol used to dilute the oil of fennel helps diffusion through the agar.

Although Deans and Richie (1987) do not consider oil of fennel as having either potent or broad-spectrum antimicrobial properties, other studies which have investigated the properties of components of oil of fennel (such as anethole) have found considerable activity against food-borne bacteria and yeast. Karapinar and Aktug (1987) found that anethole has an MIC of 75-100  $\mu\text{g ml}^{-1}$  against *S. typhimurium*, *S. aureus* and *Vibrio parahaemolyticus*. Hitokoto and co-workers (1980) found an MIC of 200  $\mu\text{g ml}^{-1}$  against a range of toxigenic fungi. These studies may indicate that the activity of oil of fennel was underestimated by the work of Deans and Richie (1987). Other components of oil of fennel which have been shown to have some antimicrobial activity include anisaldehyde (Deans and Svoboda, 1988), pinene (Deans and Svoboda, 1988; Deans and Svoboda, (1989) and (+)- $\alpha$ -phellandrene (Deans and Svoboda, 1988). These authors also identified camphene as having little or no antimicrobial activity. However, it should be noted that many of the constituents of oil of fennel have not been tested and their antimicrobial activity remains unknown.

Parabens are the alkyl esters of p-hydroxybenzoic acid. They are well known antimicrobial agents in toiletry products (Hugo and Russell, 1992) and to a more limited extent in foods (Davidson, 1993). The Preservative in Foods Regulations (1989) permits the use of the methyl, ethyl and propyl paraben in food but their restricted use may be as a result of their relatively high cost (Davidson, 1993). Even so, they have greater overall value than other "pH sensitive" food preservatives such as benzoic acid and sorbate. This is due to their high inhibitory activity at neutral pH, and the pH-activity relationship of parabens not being dependent on the dissociation of the compound (Eklund, 1985; Davidson, 1993). The antimicrobial activity of parabens has been evaluated against a wide range of Gram-positive and Gram-negative bacteria, yeast and fungi (Table 4.1). These studies have shown that antimicrobial activity increases with increasing alkyl chain length (Oka, 1960).



**Table 4.1:** Inhibitory concentrations of parabens ( $\mu\text{g ml}^{-1}$ ) against bacteria and fungi.

Adapted from Davidson (1993).

Organism	methyl	ethyl	propyl	butyl	heptyl
<i>Aeromonas hydrophila</i>	550	-	100	-	-
<i>Bacillus.Cereus</i>	1000-2000	830-1000		-	12
<i>B. megaterium</i>	1000	-	320	100	-
<i>B. subtilis</i>	1980-2130	1000-1330	250-450	63-115	-
<i>Clostridium botulinum</i>	1000-1200	800-1000	200-400	200	-
<i>Enterobacter aerogenes</i>	2000	1000	1000	4000	-
<i>Escherichia coli</i>	1200-2000	1000-2000	400-1000	1000	-
<i>Klebsiella pneumoniae</i>	1000	500	250	125	-
<i>Lactococcus lactis</i>	-	-	400	-	12
<i>Listeria monocytogenes</i>	1430-1600	-	512	-	-
<i>Micrococcus spp.</i>	-	60-110	10-100	-	-
<i>Pseudomonas aeruginosa</i>	4000	4000	8000	8000	-
<i>P. fluorescens</i>	1310	-	670	-	-
<i>P. fragi</i>	-	-	4000	-	-
<i>P. putida</i>	450	-	-	-	-
<i>Sacina lutea</i>	4000	1000	400-500	125	12
<i>Staphylococcus aureus</i>	1670-4000	1000-2500	350-540	120-200	12
<i>Streptococcus faecalis</i>	-	130	40	-	-
<i>Salmonella typhosa</i>	2000	1000	1000	1000	-
<i>S. typhimurium</i>	-	-	180->300	-	-
<i>Vibrio parahaemolyticus</i>	-	-	50-100	-	-
<i>Yersinia enterocolitica</i>	350	-	-	-	-
<i>Alternaria spp.</i>	-	-	100	-	50-100
<i>Aspergillus flavus</i>	-	-	200	-	-
<i>Aspergillus niger</i>	1000	400-500	200-250	125-200	-
<i>Byssoschlamys fulva</i>	-	-	200	-	-
<i>Candida albicans</i>	1000	500-1000	125-250	125	-
<i>Debaryomyces hansenii</i>	-	400	-	-	-
<i>Penicillium digitatum</i>	500	250	63	<32	-
<i>Penicillium chrysogenum</i>	500	250	125-200	63	-
<i>Rhizopus nigricans</i>	500	250	215	63	-
<i>Saccharomyces bayanus</i>	930	-	220	-	-
<i>Saccharomyces cerevisiae</i>	1000	500	125-200	32-200	25-100
<i>Torula utilis</i>	-	-	200	-	25
<i>Torulaspora delbrueckii</i>	-	700	-	-	-
<i>Zygosaccharomyces bailii</i>	-	900	-	-	-
<i>Zygosaccharomyces bisporus</i>	-	400	-	-	-
<i>Zygosaccharomyces rouxii</i>	-	700	-	-	-



There are no documented cases of oil of fennel being present as a constituent of any preservative combinations. In contrast, parabens have been shown to form a number of combination antimicrobial preparations (Hugo and Foster, 1963). Those which are synergistic are shown in Table 4.2. The most important of these combinations (Phenonip) is sold commercially as a preservative for cosmetics and pharmaceuticals (Denyer *et al*, 1985 citing Parker *et al*, 1968; and Wallhäuser, 1984).

Since oil of fennel and parabens have antimicrobial properties and are not prohibited as preservatives in foods under the Preservative in Foods Regulations (1989), it is conceivable that they may be used either as single agents or in combination to preserve enteral nutrient solutions. Consequently, the aims of this chapter are:-

- To evaluate the use of Tween 80 as an aid to recovery of bacterial cells from antimicrobial assays.
- To investigate the possibility of inhibiting the growth of microorganisms in enteral nutrient solutions using oil of fennel, parabens and/or combinations of oil of fennel and parabens.
- To measure the extent of synergy between oil of fennel and parabens.

**Table 4.2:** Synergistic combinations of antimicrobials containing paraben.

Component of synergistic combination other than paraben.	Reference.
Other paraben	Gerrard <i>et al</i> (1962)
Dehydroacetic acid	Winkler (1955)
Weak acids (Lactic, proprionic etc.)	Rehm and Stahl (1960)
Boric acid	
NN"-methylene bis(5'-hydroxymethyl- 2,5-dioxo-4-imidazolidinyl urea (Germall)	Berke and Rosen (1970) Jacobs <i>et al</i> (1975)
2-bromo-2-nitropropan-1,3-diol (Bronopol)	Rehm and Stahl (1960)
2-phenylethanol (Phenonip)	Parker <i>et al</i> , (1968) Richards and McBride (1971) Wilkinson (1975)
2-phenoxyethanol	Boehm (1968)
propylene and hexylene glycols	Blanchard (1980) Prickett <i>et al</i> , (1961)
sodium sulphite	Rehm and Stahl (1960)

Adapted from Denyer *et al* (1985).

## 4.2 MATERIALS AND METHODS

### 4.2.1 Materials

Essential oils were obtained from F.D. Copeland and Sons Limited, Colanor House, Westfield Street, London, UK. Oil of fennel was used neat or as a 2% solution in 95% analytical grade ethanol, where the concentrations required in broth were less than 0.03%. Methyl, ethyl and propyl parabens were purchased from Sigma Ltd and prepared fresh on the day of use as a 20% stock solution in 95% analytical grade ethanol.

### 4.2.2 Methods

#### 4.2.2.1 Determination of a suitable agent to prevent carryover of antimicrobial agents into microbial enumeration media during antimicrobial assays

Tween 80 is a non-ionic surfactant which is sometimes added to the diluent and agar in antimicrobial assays to prevent the carryover of antimicrobial agents onto the enumeration media, thus affecting bacterial counts. 10 ml quantities of nutrient broth were supplemented with 0.1% oil of fennel and inoculated with *K. aerogenes* from an overnight culture (Section 2.2.2) to give a concentration of approximately 5.00 log cfu ml<sup>-1</sup>. Tubes were incubated at 25°C over a 24 hour period and samples were taken for enumeration of bacteria at regular intervals. Bacteria were enumerated using the spread plate method (Jay, 1992).

*K. aerogenes* was grown in the presence of 0.1% oil of fennel over a period of 24 hours. Samples were taken for enumeration at regular intervals and diluted in 0.01M PBS or 0.01M PBS supplemented with 1% Tween 80 and plated onto nutrient agar or nutrient agar supplemented with 1% Tween 80.

#### 4.2.2.2 Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)

The efficacies of oil of fennel and parabens in laboratory broth and enteral nutrient solutions were evaluated by determining Minimum Inhibitory Concentrations (MIC) and the Minimum Bactericidal Concentration (MBC) using a modification of the Broth Dilution Technique as described by Miles and Amyes (1996).

##### 4.2.2.2a Setting up tubes for determining MIC and MBCs

Sterile laboratory broth or enteral nutrient solution (10 ml) was supplemented with either oil of fennel or parabens to give final concentrations ranging from 0.005% to 1% and 0.01% to 0.3% respectively. Each tube was vortexed for 30 seconds to give a fine and stable dispersion of oil droplets and then inoculated with 10 $\mu$ l of an overnight culture (See section 2.2.2). The inocula used gave bacterial concentrations of approximately 5.00 log cfu ml<sup>-1</sup> and yeast concentrations of approximately 4.00 log cfu ml<sup>-1</sup>. Tubes containing broth or Osmolite without antimicrobials were included as a control in each experiment. Tubes were incubated at 25°C for 24 hours to simulate the "hanging" conditions of enteral feeding systems.

##### 4.2.2.2b Reading results from assays to determine MIC and MBC

MIC and MBC were assessed via two different procedures modified from the methods described by Miles and Amyes (1996). The choice of procedure depended on the growth medium selected for use.

Tubes from experiments conducted in laboratory media were examined visually for growth of microorganisms. The MIC was defined as the *least amount of antimicrobial agent that inhibited visible growth of an organism after 24 hours culture*. Tubes that had no visible growth were sub-cultured onto agar plates (100 $\mu$ l per plate). Thus, the MBC was defined as the *least amount of an antimicrobial agent required to prevent growth after sub-culture onto antimicrobial agent free media*.

In assays conducted in Osmolite, visual observations of growth could not be made due to the opacity of the Osmolite. Therefore, inhibition of microorganisms was measured by making  $10^2$  dilutions in 0.01M PBS and sub-culturing onto agar plates (100 $\mu$ l per plate). In addition, 100  $\mu$ l of undiluted material from each tube was sub-cultured directly onto agar plates. The MIC was defined as the *least amount of antimicrobial agent required to inhibit growth in tubes incubated at 25°C for 24 hours, so that numbers were approximately equal to or less than that of the original inoculum*. The MBC was defined as the *least amount of antimicrobial agent required to prevent re-growth of microorganisms after sub-culture onto antimicrobial agent free media*.

All plates were incubated at 37°C for 18 hours (bacteria) or 25°C for 36 hours (yeast) to determine the presence of viable microorganisms. Results were expressed as the percentage concentration of antimicrobial agent in growth media.

#### 4.2.2.3 Checkerboard

The term "checkerboard" refers to the pattern of tubes containing different concentrations of two antimicrobials being tested. Thus, a tube may contain no antimicrobial agent, a concentration of one antimicrobial agent or a combination of both antimicrobial agents. A range of concentrations of each antimicrobial agent are used so that the antimicrobial effect of a large numbers of permutations are assessed.

##### 4.2.2.3a Preparation of checkerboards

The combinations of oil of fennel and methyl paraben used were based on twofold multiples of the MIC defined in Section 4.2.2. However, in cases where the MIC was found to exceed the maxima (>1% oil of fennel or 0.3-0.4% methyl paraben), multiples of these maxima were used in the Checkerboard Assays. To determine both the antimicrobial activity of combinations, and to determine the presence of synergy, tubes of laboratory media and Osmolite (10 ml) were supplemented with oil of fennel

and methyl paraben (Section 4.2.1). In each experiment a set of controls was incorporated. This included a tube containing no antimicrobial agents, and a set of tubes with just one of the antimicrobial agents at each concentration used in the experiment. All tubes were vortexed for 30 seconds to suspend the oil of fennel or dissolve the methyl paraben and inoculated to give approximately  $5.00 \log \text{ cfu ml}^{-1}$  of a bacterial strain or approximately  $4.00 \log \text{ cfu ml}^{-1}$  of yeast from overnight cultures (Section 2.2.2). Tubes were incubated at  $25^{\circ}\text{C}$  for 24 hours and results were read as outlined in the following sections.

#### 4.2.2.3b Collecting Checkerboard Assay results

Results were read using two procedures, depending on the growth media used, modified from those of Eliopoulous and Moellering (1991). This was because experiments conducted in laboratory media could be read visually while the opacity of Osmolite meant growth/inhibition could only be detected by plate counts.

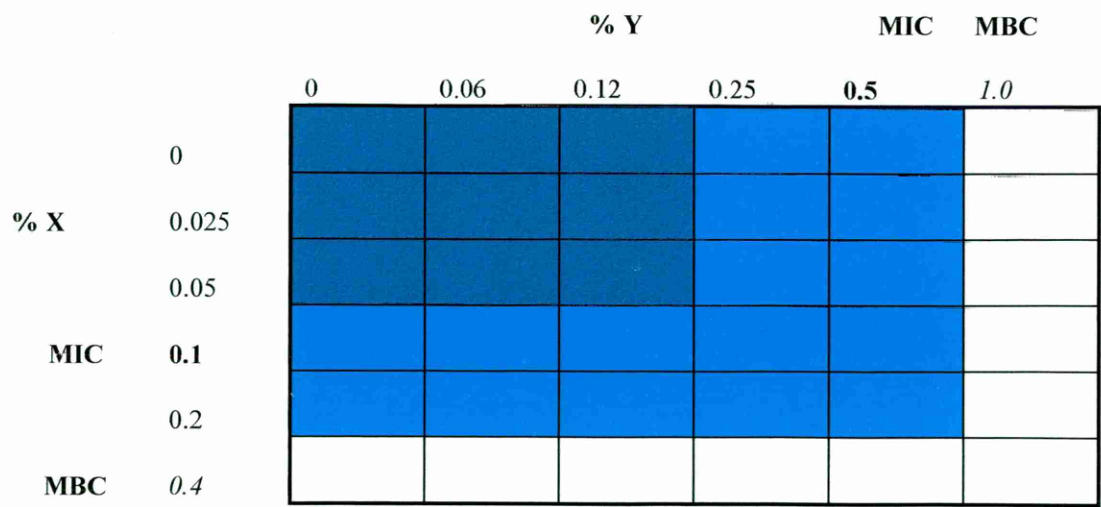
To determine growth or inhibition of microorganisms in laboratory media, a visual assessment of growth was made and the results noted. Tubes which had no visible growth were sub-cultured onto agar plates (100 $\mu\text{l}$  per plate). In experiments conducted with Osmolite, inhibition was assessed by making  $10^2$  or  $10^3$  dilutions in 0.01M PBS and spreading 100 $\mu\text{l}$  onto agar plates. In addition, 100 $\mu\text{l}$  of undiluted material was taken from each tube and spread directly onto a second agar plate. All plates were incubated either at  $37^{\circ}\text{C}$  for 18 hours (bacteria) or  $25^{\circ}\text{C}$  for 24 hours (yeast).

#### 4.2.2.3c: Interpretation of Checkerboard Assay results

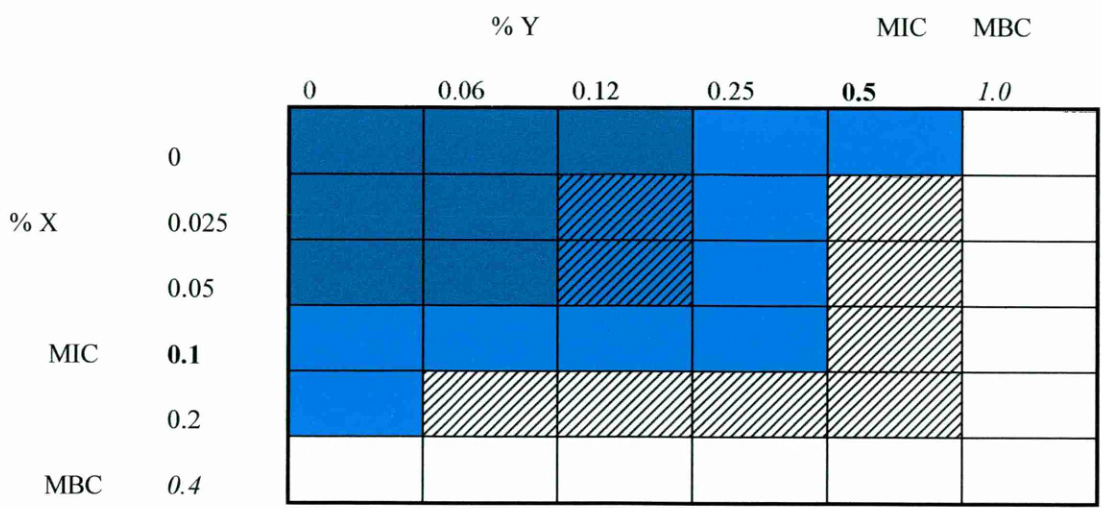
The results of Checkerboard Assays were interpreted using the criteria shown in Table 4.3 and results were presented in table-like figures (Figures 4.1a and b). Each box represents the results of growth from a tube containing a concentration of a single antimicrobial agent or a combination of agents. In Figure 4.1a inhibition by

**Table 4.3:** Criteria for assessing the activity of antimicrobial combinations of oil of fennel and methyl paraben.

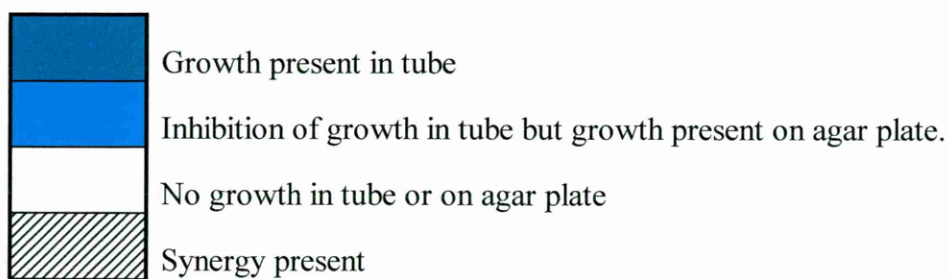
	Laboratory Media	Osmolite.
Category 1: No apparent activity.	Observable growth in test-tube.	Uncountable numbers of colonies on agar plate of $10^2$ or $10^3$ dilution.
Category 2: Inhibitory activity or weak cidal activity.	No observable growth in tube but uncountable colonies on agar plate spread with 100 $\mu$ l sample from test-tube.	Countable colonies on an agar plate spread with 100 $\mu$ l of a $10^2$ dilution of a test-tube.
Category 3: Strong cidal activity.	Countable colonies or no colonies on an agar plate spread with 100 $\mu$ l sample from a test-tube.	Countable colonies or no colonies on agar plate spread with a 100 $\mu$ l sample from a test-tube.



**Figure 4.1a:** Checkerboard showing no synergism between antimicrobial agents



**Figure 4.1b:** Checkerboard showing a synergistic relationship between antimicrobial agents





agents is shown which is not synergistic but merely additive, while in Figure 4.1b inhibition of the microbe is synergistic. A synergistic antimicrobial effect was deduced from results by comparing the addition of effects in the control tubes with the effect in the tube containing the corresponding combinations. Synergistic combinations were defined as *those which had an effect 1.00 log cfu ml<sup>-1</sup> more than the addition of the effects in the corresponding control tubes*. Marginal cases were designated and identified in figures as "possible synergy".

#### 4.2.2.4 Killing Curves

Killing curve assays were used to determine a dynamic picture of the antimicrobial activity of combinations of oil of fennel and methyl paraben. Tubes supplemented with oil of fennel and paraben (Section 4.2.2) were vortex-mixed for 30 seconds to ensure a fine suspension of oil of fennel droplets. Each set of Killing Curve Assays included a control containing no antimicrobial agents. Tubes were inoculated to give approximately 5.00 log cfu ml<sup>-1</sup> of a bacterial strain or approximately 4.00 log cfu ml<sup>-1</sup> of yeast from an overnight culture (Section 2.2.2). All tubes were incubated at 25°C and sampled at intervals over a 24 hour period. Bacteria were enumerated using the Spread Plate Method as described by Jay (1992). Agar plates were incubated at 37°C for 18 hours (bacteria) or at 25°C for 36 hours (yeast). Results were expressed as the mean of at least 3 experiments with its SEM and compared using Student's t-test and ANOVA. Synergistic combinations were defined as *those which had an antimicrobial effect 1.00 log cfu ml<sup>-1</sup> more than the addition of effects when each agent was used singly*.

## **4.3 RESULTS**

### **4.3.1. Use of Tween 80 as a suitable agent to prevent carryover of antimicrobial agents into microbial enumeration media during antimicrobial assays**

The efficacy of a non-ionic surfactant (Tween 80) at preventing “carryover” of oil of fennel during antimicrobial assays was evaluated, over 24 hours, in either diluent, agar or in both (Table 4.4). The objective of this experiment was to ascertain whether carryover decreased the recovery of viable organisms. Results showed that a 1% concentration of Tween in either the diluent, the agar or both of these did not significantly increase the number of bacteria recovered, compared to a control without Tween 80. Therefore, it was decided that there was no benefit in adding Tween 80 to either diluent or agar in order to prevent carryover of oil of fennel during enumeration and it was omitted from subsequent experiments.

### **4.3.2 Minimum inhibitory concentrations and minimum bactericidal concentrations**

The inhibitory, bactericidal and fungicidal properties of various antimicrobial agents were evaluated by measuring the MIC and MBC in laboratory media and in a milk-based enteral nutrient solution (Osmolite) against several strains of bacteria and one strain of yeast, known to contaminate enteral nutrient solutions. Results are shown in Tables 4.5 and 4.6.

#### **4.3.2.1 MIC and MBC of oil of fennel in laboratory media and Osmolite**

Growth of microbial strains in laboratory media was inhibited by concentrations of oil of fennel in the range 0.008->1%. As one would expect, the corresponding cidal concentrations were much greater and in the range 0.015->1%. Three of the microbial strains tested had MBCs greater than the highest concentration of oil of fennel tested in experiments.

**Table 4.4:** Evaluation of Tween 80 as a neutralizing agent for preventing carry over of oil of fennel on to enumeration media.

Time	0 hour	1 hour	2 hours	4 hours	8 hours	24 hours	significance *
Control	5.52±0.009	3.01±0.054	1.70±0.055	1.00±0	1.00±0	1.00±0	
Tween 80 in agar	5.52±0.012	2.88±0.09	1.52±0.175	1.00±0	1.00±0	1.00±0	p=>0.05
Tween 80 in PBS	5.58±0.092	3.18±0.043	1.66±0.140	1.00±0	1.00±0	1.00±0	p=>0.05
Tween 80 in agar and PBS.	5.56±0.003	3.18±0.031	1.58±0.154	1.00±0	1.00±0	1.00±0	p=>0.05

\* Comparison of killing curves with control using ANOVA

Figures represent the mean of three independent results expressed in log cfu ml<sup>-1</sup> with SEM.

**Table 4.5:** Minimum Inhibitory Concentrations of oil of fennel and parabens in laboratory media and milk-based enteral nutrient solutions.

	Media	% Oil of fennel	% methyl parabens	% ethyl parabens	% propyl parabens
<i>C. albicans</i>	SAB	0.135	0.07	0.05	0.03
<i>E. coli</i>	NB	0.1	0.08	0.075	0.05
<i>Ent. cloacae</i>	NB	>1	0.075	0.05	0.05
<i>K. aerogenes</i>	NB	0.008	0.075	0.075	0.05
<i>P. aeruginosa</i>	NB	>1	0.15	0.125	0.075
<i>S. aureus</i>	TSB	0.125	0.125	0.075	0.05
<i>C. albicans</i>	Osmolite	0.9	0.15	-	-
<i>E. coli</i>		>1	0.3	-	-
<i>Ent. cloacae</i>		>1	0.4	-	-
<i>K. aerogenes</i>		>1	0.3	-	-
<i>P. aeruginosa</i>		>1	>0.3	-	-
<i>S. aureus</i>		>1	>0.3	-	-

NB: Nutrient Broth, TSB: Tryptone Soya Broth, SAB: Sabouraud Broth. Each figure represents the modal percentage concentration of an antimicrobial agent from at least three independent experiments.

**Table 4.6:** Minimum Bactericidal Concentration of oil of fennel and parabens in laboratory media and milk-based enteral nutrient solutions.

	Media	% Oil of fennel	% methyl parabens	% ethyl parabens	% propyl parabens
<i>C. albicans</i>	SAB	0.5	>0.3	>0.3	0.075
<i>E. coli</i>	NB	0.3	0.3	0.2	0.075
<i>Ent. cloacae</i>	NB	>1	0.3	0.15	0.075
<i>K. aerogenes</i>	NB	0.015	0.3	0.2	0.07
<i>P. aeruginosa</i>	NB	>1	0.25	>0.2	>0.2
<i>S. aureus</i>	TSB	>1	0.3	0.3	0.2
<i>C. albicans</i>	Osmolite	>1*	0.4		
<i>E. coli</i>		>1*	>0.4		
<i>Ent. cloacae</i>		>1*	>0.4		
<i>K. aerogenes</i>		>1*	>0.4		
<i>P. aeruginosa</i>		>1*	>0.3		
<i>S. aureus</i>		>1*	>0.3		

NB: Nutrient Broth, TSB: Tryptone Soya Broth, SAB: Sabouraud Broth. Each figure represents the modal percentage concentration of an antimicrobial agent from at least three independent experiments.

The concentrations of oil of fennel required to inhibit the growth of microbial strains in Osmolite were much greater than in laboratory media. The results showed that none of the bacteria growing in Osmolite were inhibited by the maximum concentration of oil of fennel used in experiments (1%). However, *C. albicans* was more susceptible to oil of fennel and was inhibited by a concentration of 0.9%. No MBC were found in the range of oil of fennel concentrations tested.

#### 4.3.2.2 MIC and MBC of parabens in laboratory media and Osmolite

Microorganisms growing in laboratory media were inhibited by concentrations of methyl parabens in the range of 0.07-0.15% and by concentrations of ethyl parabens in the range of 0.05-0.125%. However, the most effective form of paraben was the propyl derivative which inhibited organisms in the range 0.03-0.075%. Concentrations of methyl parabens required to kill microbial strains in laboratory media were 0.25-<0.3, while ethyl parabens was slightly more effective with MBCs in the range 0.15-<0.3%. However, propyl parabens was also the most effective form of paraben at killing organisms with MBCs in the range 0.05-<0.2%.

Concentrations of 0.15-<0.3% methyl parabens were inhibitory to the growth of microorganisms growing in Osmolite. None of the bacterial strains were killed by the concentrations of methyl parabens used in these experiments. However, 0.4% methyl parabens was cidal to *C. albicans* growing in Osmolite. MIC and MBCs were not measured for ethyl and propyl parabens because time constraints prevented the experiments being carried out.

### **4.3.3 The antimicrobial properties of combinations of oil of fennel and paraben in laboratory media and milk-based enteral nutrient solutions**

Control tubes contained only one antimicrobial agent. In all experiments the results from these control tubes were found to agree with previous experiments determining the Minimum Inhibitory Concentrations and the Minimum Bactericidal Concentrations.

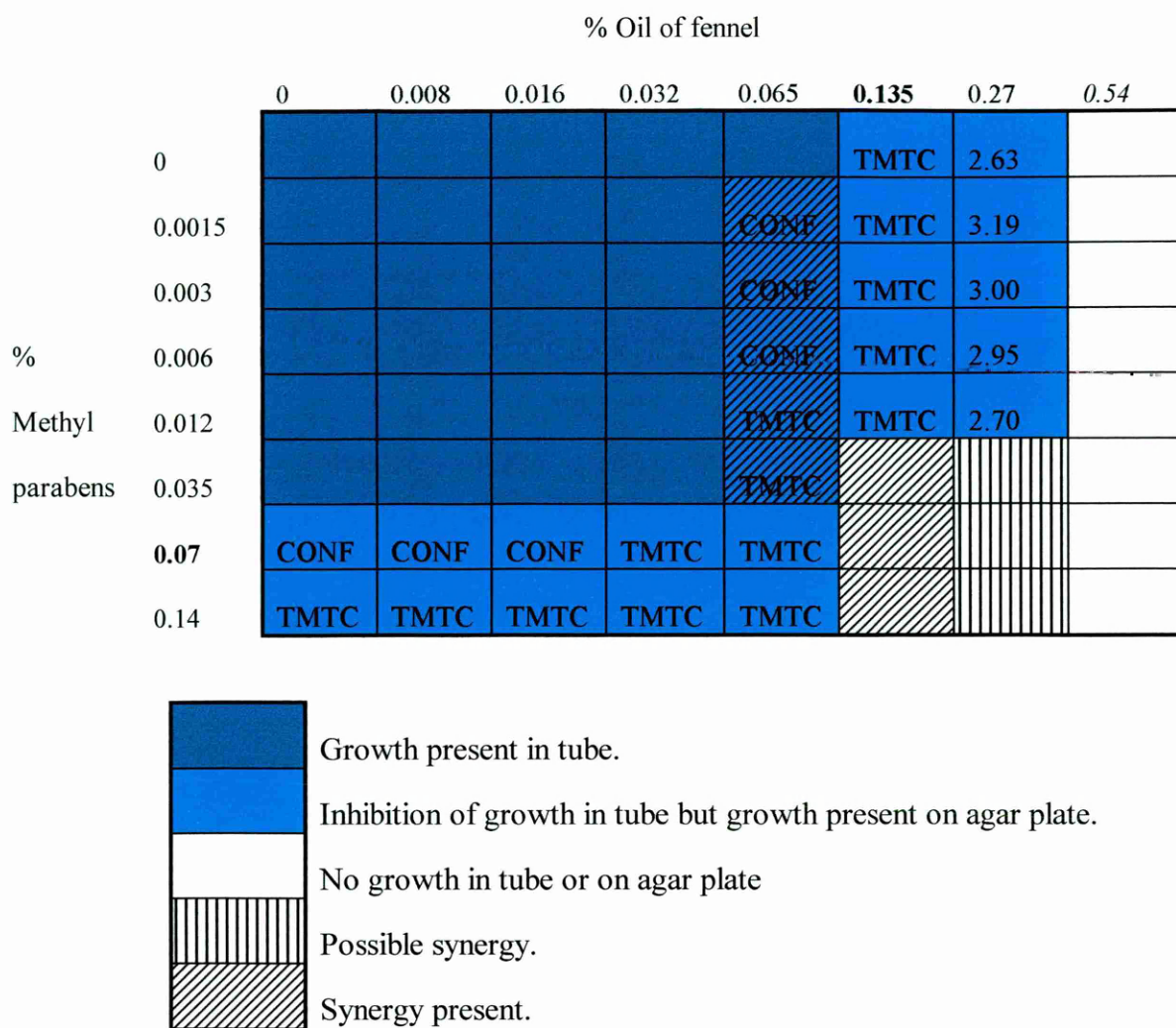
#### **4.3.3.1 *C. albicans***

Sabouraud broth was supplemented with combinations of 0.008-0.54% oil of fennel and 0.0015-0.14% methyl parabens (Figure 4.2). Out of a total of 49 tubes, 15 of the combinations had no apparent antimicrobial effect. A further 21 combinations had an inhibitory or weak cidal activity and the remaining 13 combinations had strong cidal activity against *C. albicans*. Three of these combinations were synergistic and a further three were possibly synergistic. Synergy was observed with combinations formed from 0.065% oil of fennel and 0.015%-0.035% methyl parabens and 0.135% oil of fennel and 0.035-0.14% methyl parabens.

Osmolite was supplemented with combinations of 0.05-0.9% oil of fennel and 0.0012-0.2% methyl parabens (Figure 4.3). Fourteen combinations had no apparent antifungal activity against *C. albicans*, 11 combinations were inhibitory or weakly cidal and none were strongly cidal. None of the combinations were synergistic.

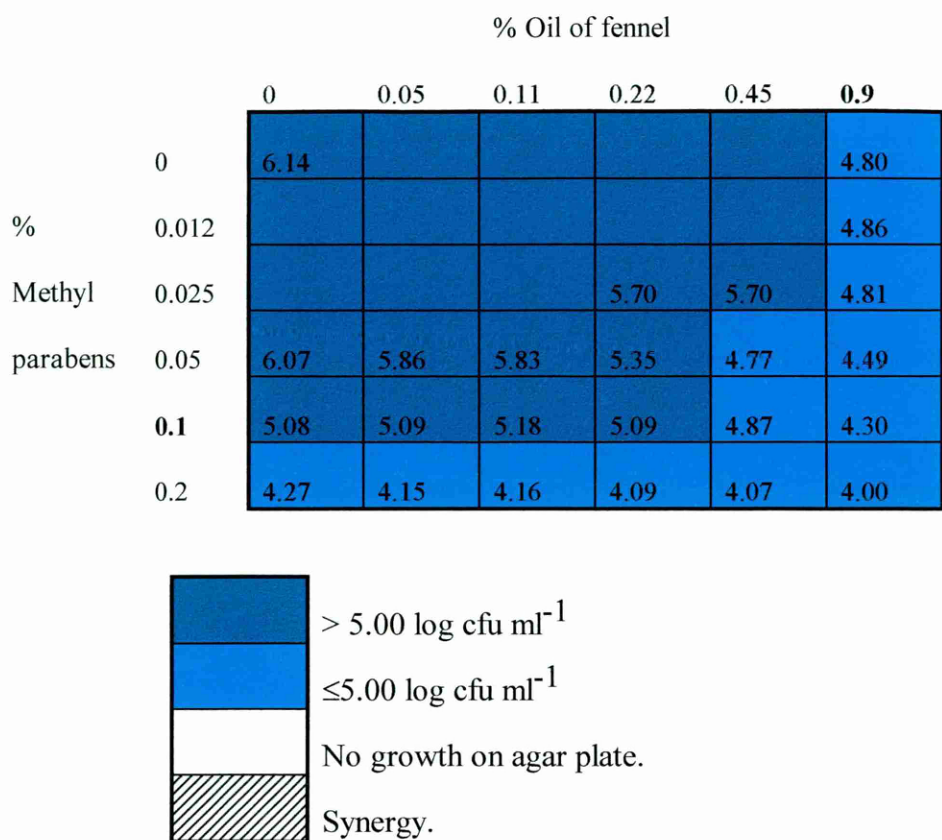
#### **4.3.3.2 *E. coli***

25 tubes of nutrient broth were supplemented with combinations of 0.012-0.2% of oil of fennel and 0.005-0.08% methyl parabens (Figure 4.4). Eight of these combinations had no apparent inhibitory activity, another 8 combinations had an inhibitory or weak bactericidal activity and the remaining nine combinations had strong bactericidal activity.

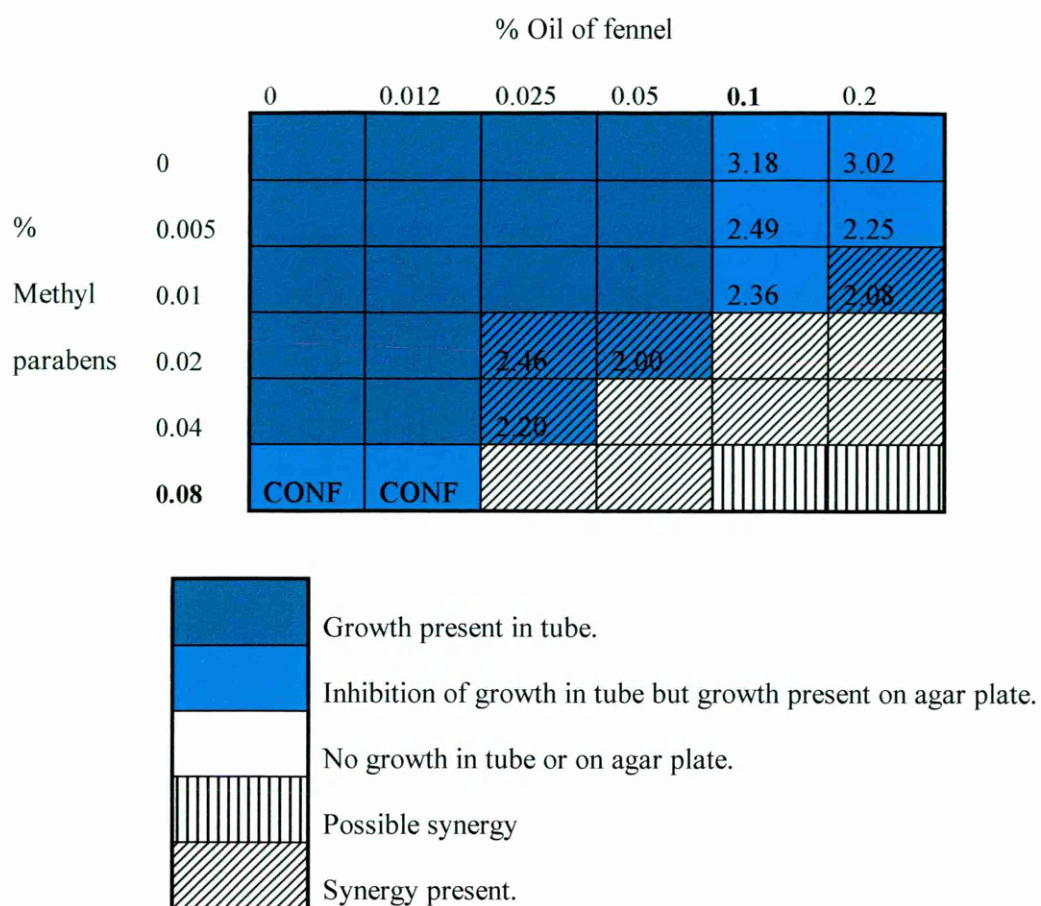


**Figure 4.2:** Checkerboard assay of oil of fennel and methyl parabens activity against *C. albicans* in laboratory media. CONF: Confluent growth on agar plate. TMTC: Too many colonies to count on agar plate. Emboldened figures represent the MIC of each antimicrobial. Figures in *italics* represent the MBC of the antimicrobial agents. Figures in boxes represent mean microbial counts from three plates in log cfu ml<sup>-1</sup>.

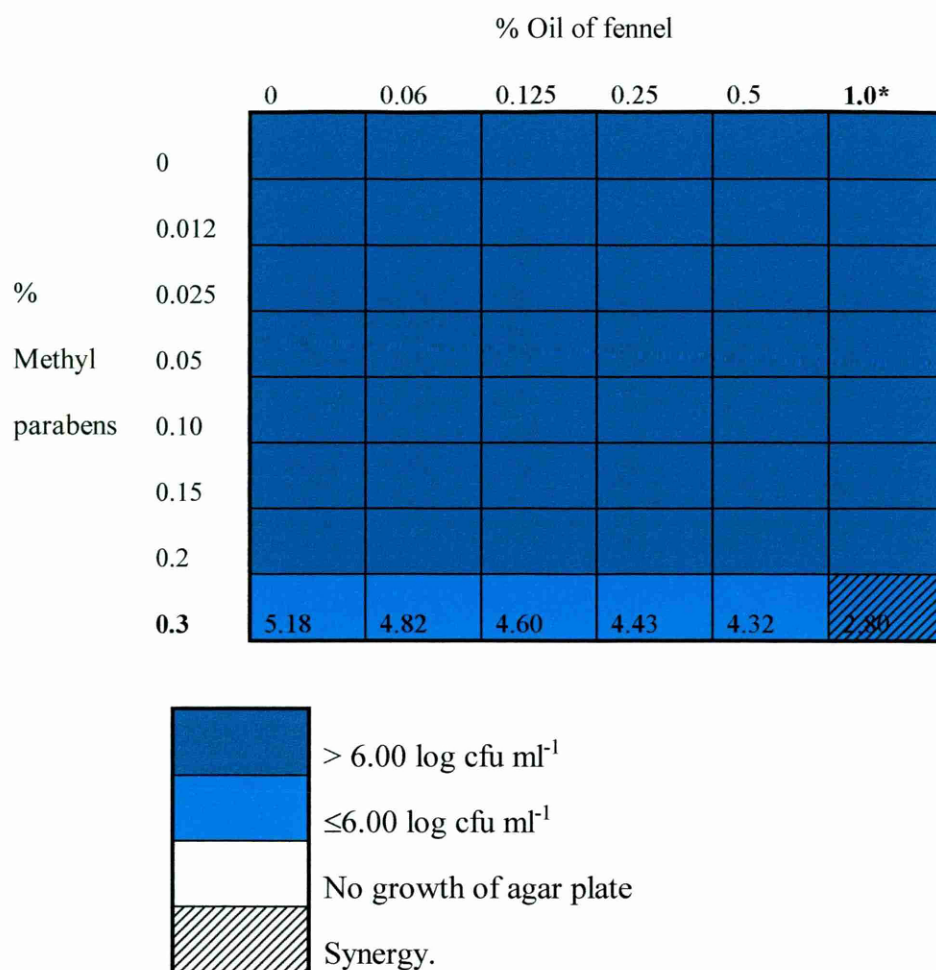




**Figure 4.3:** Checkerboard assay of oil of fennel and methyl parabens activity against *C. albicans* in Osmolite. Emboldened figures represent the MIC of each antimicrobial agent. Figures in boxes represent mean microbial counts from three plates in log cfu ml<sup>-1</sup>.



**Figure 4.4:** Checkerboard assay of oil of fennel and methyl parabens activity against *E. coli* in laboratory media. CONF: Confluent growth on the agar plate. Emboldened figures represent the MIC of each antimicrobial agent. Figures in boxes represent mean microbial counts from three plates in log cfu ml<sup>-1</sup>.



**Figure 4.5:** Checkerboard assay of oil of fennel and methyl parabens activity against *E. coli* in Osmolite. Emboldened figures represent the MIC of each antimicrobial agent. \* MIC (oil of fennel). Figures in boxes represent mean microbial counts from three plates in log cfu ml<sup>-1</sup>

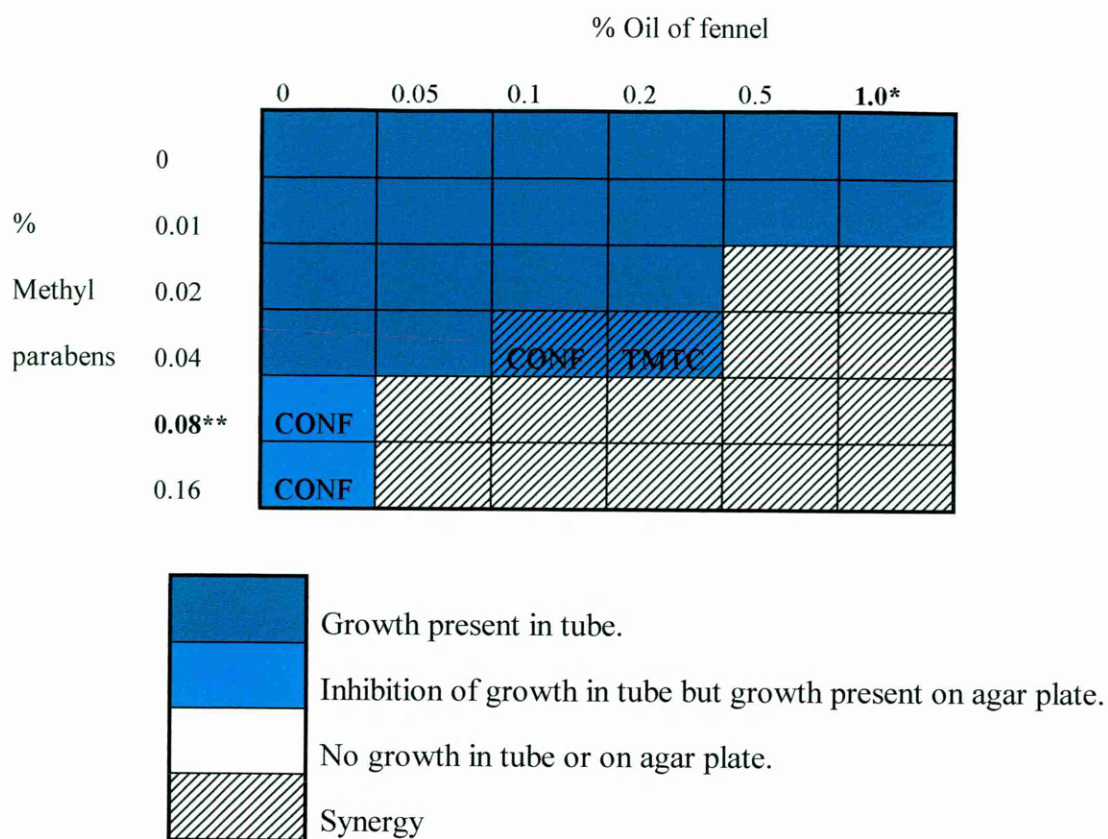
Bactericidal combinations all contained concentrations of methyl parabens in the range 0.02-0.08% but had a wide range of concentrations of oil of fennel (0.025-0.2%). Eleven of the combinations showed synergism and a further two were possibly synergistic. Four of the synergistic combinations were inhibitory or weakly cidal and the other seven synergistic combinations were strongly cidal against *E. coli*.

Osmolite was supplemented with combinations of 0.06-1.0% oil of fennel and 0.012-0.3% methyl parabens (Figure 4.5). Out of a total of 35 combinations, 30 had no apparent inhibitory activity against *E. coli* growing in Osmolite. These were all the combinations which contained 0.2% or less methyl parabens. Five combinations had inhibitory or weak cidal activity against *E. coli* and these contained 0.06-1.0% oil of fennel and 0.3% methyl paraben. Only one combination 1% oil of fennel with 0.3% methyl parabens was determined as being synergistic.

#### 4.3.3.3 *Ent. cloacae*

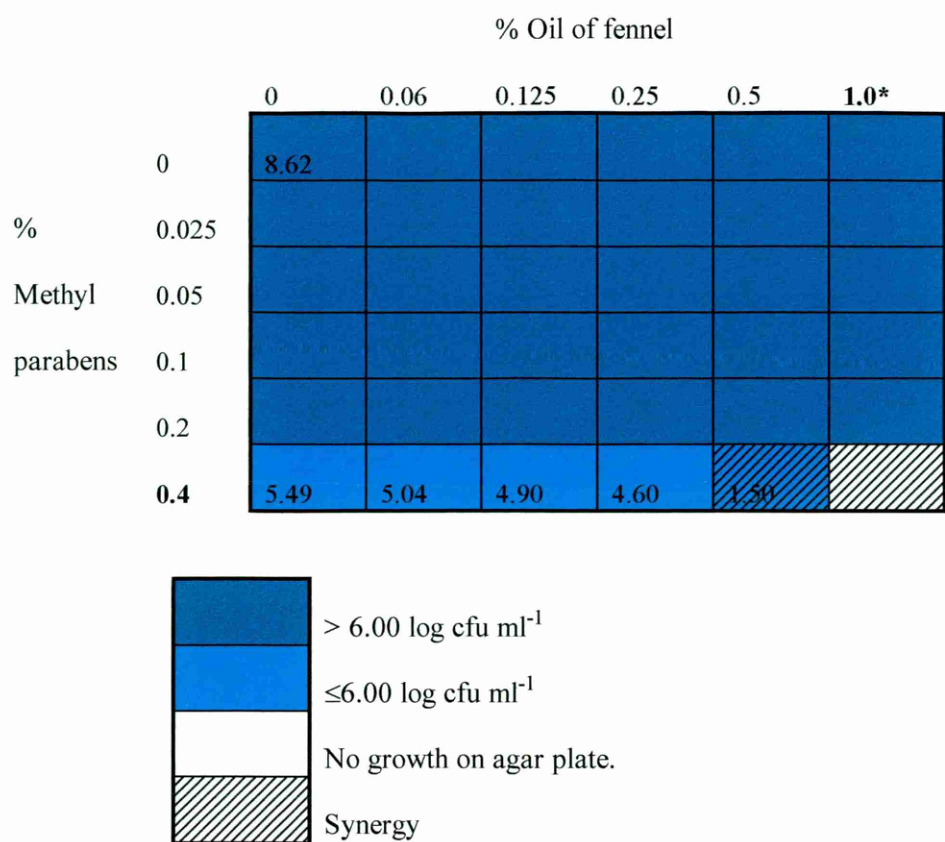
Nutrient broth was supplemented with combinations of 0.05-1.0% oil of fennel and 0.01-0.16% methyl parabens (Figure 4.6). Out of a total of 25 combinations, nine had little or no apparent inhibitory activity against *Ent. cloacae* in laboratory media, two combinations had inhibitory or weak cidal activity and 14 combinations had strong bactericidal activity. Sixteen of these combinations were synergistic. Two synergistic combinations were inhibitory or weakly cidal and the remaining 14 synergistic combinations were all strongly cidal.

25 tubes of Osmolite were supplemented with combinations of 0.06-1.0% oil of fennel and 0.025-0.4% methyl parabens (Figure 4.7). Twenty of the combinations had no apparent inhibitory activity against *Ent. cloacae* in Osmolite, four combinations had strong inhibitory or weak cidal activity and one combination (1.0% oil of fennel and 0.4% methyl parabens) had strong cidal activity. All the combinations which had either strong inhibitory or weak cidal activity contained a high concentration of methyl



**Figure 4.6:** Checkerboard assay of oil of fennel and methyl parabens activity against *Ent. cloacae* in laboratory media. CONF: Confluent growth on the agar plate. TMTC: Too many colonies to count on the agar plate. Emboldened figures represent the MIC of each antimicrobial agent. \* MIC (oil of fennel) =  $>1\% \pm 0.1$ . \*\* MIC (methyl parabens)  $0.075\% \pm 0.025$ .





**Figure 4.7:** Checkerboard assay of oil of fennel and methyl parabens activity against *Ent. cloacae* in Osmolite. Emboldened figures represent the MIC of each antimicrobial agent. \* MIC (oil of fennel )=>1.0%. Figures in boxes represent mean microbial counts from three plates in log cfu ml<sup>-1</sup>.

parabens (0.4%). Two combinations were synergistic (0.5% oil of fennel and 0.4% methyl parabens and 1.0% oil of fennel and 0.4% methyl parabens).

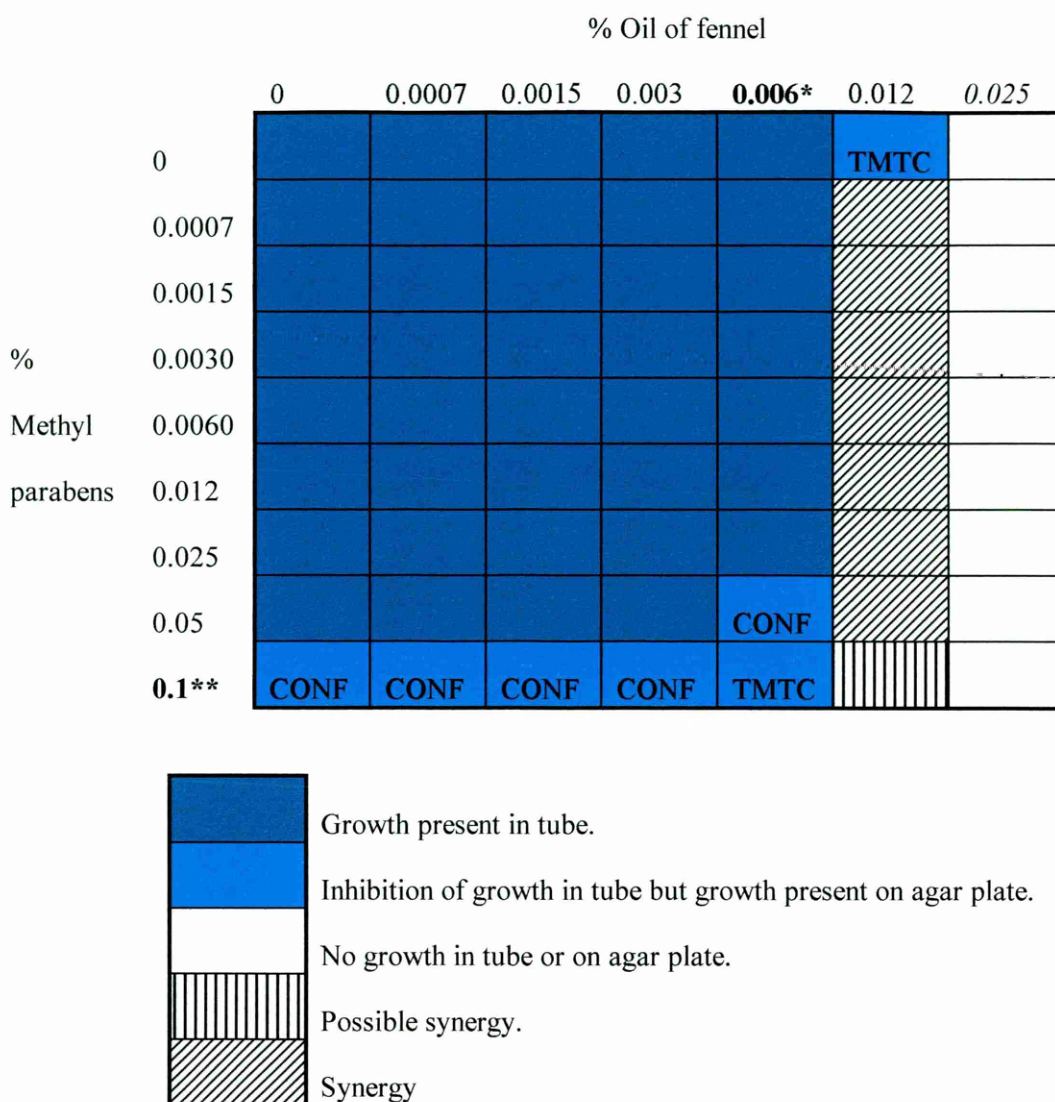
#### 4.3.3.4 *K. aerogenes*

A total of 48 tubes of nutrient broth were supplemented with combinations of 0.0007-0.025% oil of fennel and 0.0007-0.1% methyl parabens (Figure 4.8). 27 of the combinations tested had no apparent inhibitory activity against *K. aerogenes* growing in nutrient broth, 5 combinations had inhibitory or weak cidal activity and 16 combinations had a strong cidal activity. Seven combinations were synergistic. These contained 0.012% oil of fennel with concentrations of methyl parabens in the range 0.0007-0.05%. The combination of 0.012% oil of fennel and 0.1% methyl parabens was possibly synergistic.

Osmolite was supplemented with combinations of 0.06-1.0% oil of fennel and 0.025-0.4% methyl parabens (Figure 4.9). Nineteen out of a total of 25 combinations had no apparent antimicrobial activity against *K. aerogenes* growing in Osmolite. Five combinations had inhibitory or a weak cidal activity and one combination (1.0% oil of fennel and 0.4% methyl parabens) was strongly cidal. Two of these combinations were synergistic (1.0% oil of fennel and 0.2% methyl parabens and 1.0% oil of fennel and 0.4% methyl parabens).

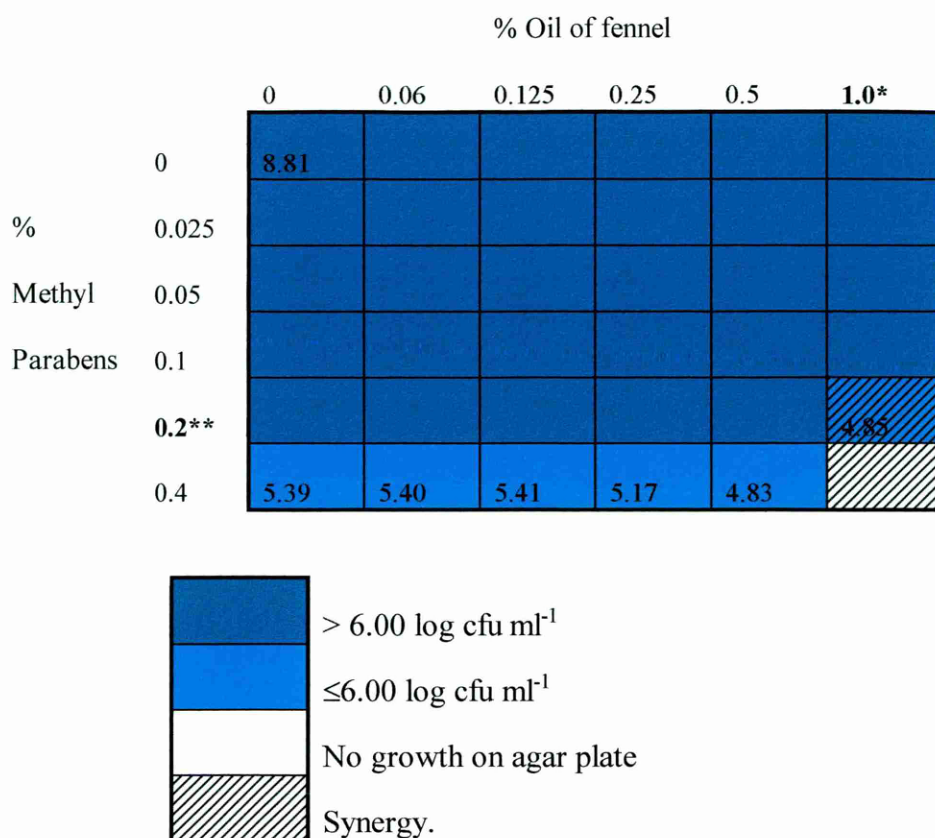
#### 4.3.3.5 *P. aeruginosa*

Nutrient broth was supplemented with combinations of 0.06-1.0% oil of fennel and 0.4-0.025% methyl parabens (Figure 4.10). Out of total of 25 combinations tested, 15 showed no apparent inhibition of *P. aeruginosa* growing in nutrient broth. No combinations had strong inhibition or weak cidal activity but 10 combinations had strong cidal activity. Five of these combinations were synergistic. These contained 0.2% methyl parabens and 0.06-1.0% oil of fennel.

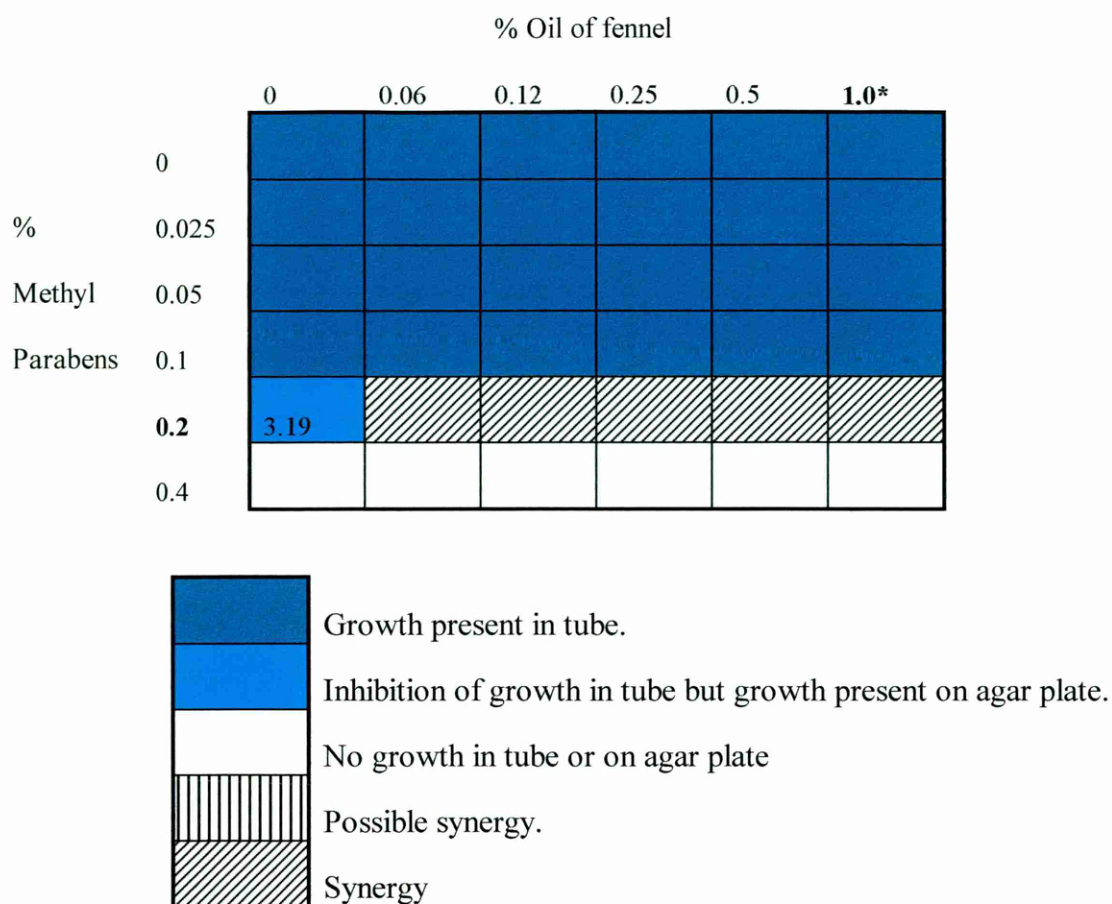


**Figure 4.8:** Checkerboard assay of oil of fennel and methyl parabens activity against in *K. aerogenes* in laboratory media. CONF: Confluent growth on the agar plate. TMTC: Too many colonies to count on the agar plate. Emboldened figures represent the MIC of each antimicrobial agent. Figures in italics represent the minimum bactericidal concentration of each antimicrobial agent. \*MIC (Oil of fennel) =0.008%, MIC (Methyl Parabens)=0.075%. Figures in boxes represent mean microbial counts from three plates in log cfu ml<sup>-1</sup>.

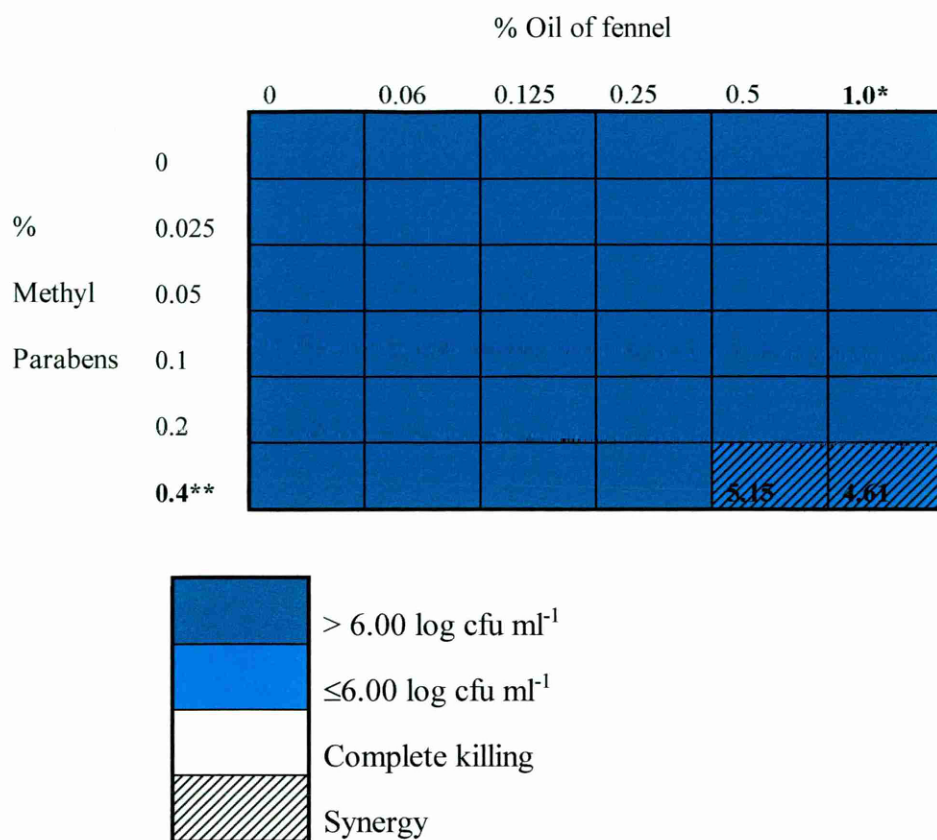




**Figure 4.9:** Checkerboard assay of oil of fennel and methyl parabens activity against *K aerogenes* in Osmolite. Emboldened figures represent the MIC of each antimicrobial agent. \* Actual MIC was >1% oil of fennel. \*\* Actual MIC was 0.3% methyl parabens. Figures in boxes represent mean microbial counts from three plates in log cfu ml<sup>-1</sup>.



**Figure 4.10:** Checkerboard assay of oil of fennel and methyl parabens activity against *P. aeruginosa* in laboratory media. Emboldened figures indicate the MIC of each antimicrobial agent. \*MIC (oil of fennel) = >1%. \*\*MIC (methyl parabens) = 0.25%. Figures in boxes represent mean microbial counts from three plates in log cfu ml<sup>-1</sup>.



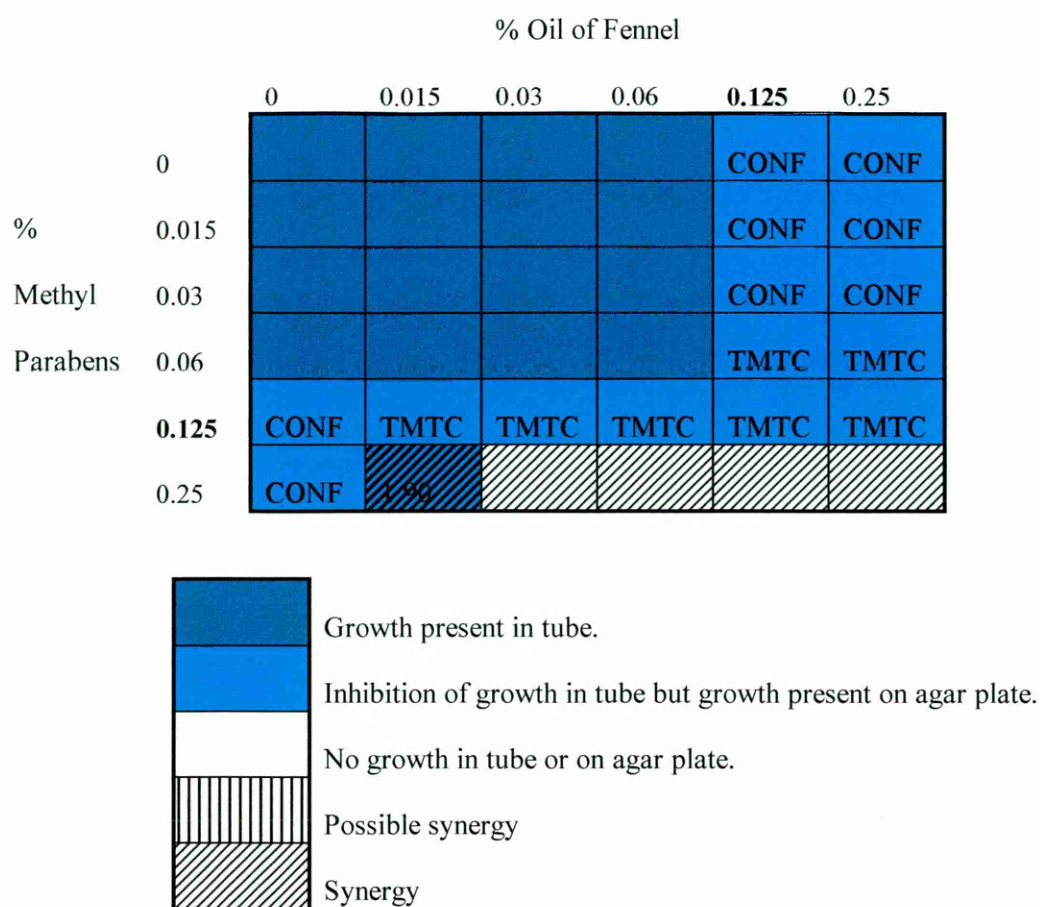
**Figure 4.11:** Checkerboard assay of oil of fennel and methyl parabens activity against *P. aeruginosa* in Osmolite. Emboldened figures represent the MIC of each antimicrobial agent. \*MIC (oil of fennel) >1%. \*\*MIC (methyl parabens) >0.4%. Figures in boxes represent mean microbial counts from three plates in log cfu ml<sup>-1</sup>.

25 tubes of Osmolite were supplemented with combinations of 0.06-1.0% oil of fennel and 0.025-0.4% methyl parabens (Figure 4.11). The vast majority of combinations (23) had no apparent inhibitory activity against the growth of *P. aeruginosa* in Osmolite. However, 2 combinations (0.5% oil of fennel and 0.4% methyl parabens, and 1.0% oil of fennel and 0.4% methyl parabens) had inhibitory or a weak cidal activity. Both these combinations were synergistic. No combinations had strong cidal activity.

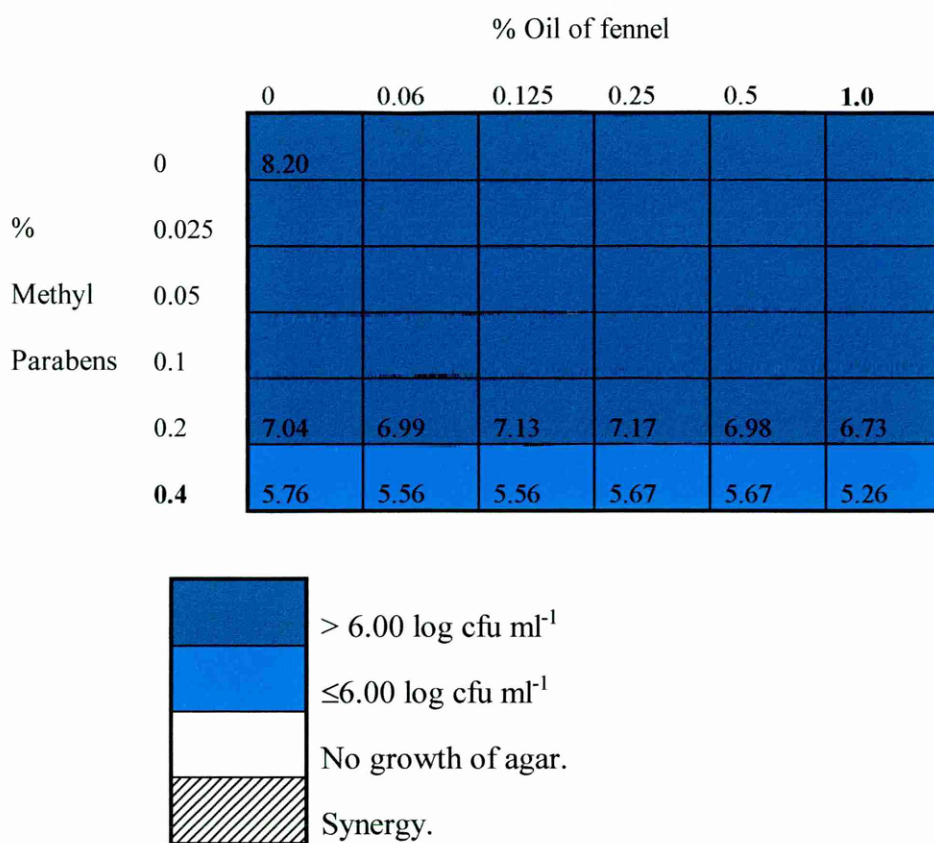
#### 4.3.3.6 *S. aureus*

Tryptone soya broth was supplemented with combinations of 0.015-0.25% oil of fennel and 0.015-0.25% methyl parabens (Figure 4.12). Nine, out of a total of 25 combinations tested, had no apparent inhibitory activity against *S. aureus* growing in tryptone soya broth, 12 tubes had inhibitory or weak cidal activity and four combinations had strong cidal concentrations. Combinations with strong cidal activity contained 0.03-0.25% oil of fennel and 0.25% methyl parabens. Five combinations were synergistic. The synergistic combinations contained 0.015-0.25% oil of fennel and 0.25% methyl parabens.

25 tubes of Osmolite were supplemented with combinations of 0.06-1.0% oil of fennel and 0.025-0.4% methyl parabens (Figure 4.13). The majority of combinations (20) had no apparent inhibitory activity against *S. aureus* growing in Osmolite, five combinations had inhibitory or weak cidal activity but no combinations had strong cidal activity. There were no synergistic combinations and all the combinations of oil of fennel and methyl parabens were less potent in Osmolite compared to laboratory media.



**Figure 4.12:** Checkerboard assay of oil of fennel and methyl parabens activity against *S. aureus* in laboratory media. CONF: Confluent growth on the agar plate. TMTC: Too many colonies to count on the agar plate. Emboldened figures represent the MIC of each antimicrobial agent. Figures in boxes represent mean microbial counts from three plates in log cfu ml<sup>-1</sup>.



**Figure 4.13:** Checkerboard assay of oil of fennel and methyl parabens activity against *S. aureus* in Osmolite. Emboldened figures represent the MIC of each antimicrobial agent. Figures in boxes represent mean microbial counts from three plates in log cfu ml<sup>-1</sup>.



#### 4.3.4 Killing Curves

The antimicrobial action of oil of fennel, parabens and combinations of these agents against *C. albicans*, *E. coli*, *K. aerogenes* and *S. aureus* was determined over a 24 hour period at 25°C in laboratory media.

##### 4.3.4.1 *C. albicans*

*C. albicans* grew rapidly in Sabouraud broth without antimicrobial agents increasing from 4.41 to 7.20 log cfu ml<sup>-1</sup> over 24 hours (Table 4.7). In the presence of 0.2% oil of fennel there was cidal action on *C. albicans* and numbers decreased from an initial inoculum of 4.28 log cfu ml<sup>-1</sup> to 2.53 log cfu ml<sup>-1</sup> over the period of the experiment. Numbers of *C. albicans* were significantly lower than in the control tube at all time intervals ( $p < 0.01$ - $0.001$ ). In contrast, in the presence of 0.1% methyl parabens numbers of *C. albicans* remained constant, with significant inhibition of growth after 4 hours ( $p < 0.01$ ). 0.1% ethyl parabens was also fungistatic against *C. albicans* with numbers not significantly changing during the experiment ( $p < 0.05$ ). However, significant inhibition of growth compared to control tubes was present at 2 hours ( $p < 0.05$ ). 0.1% propyl parabens had a cidal effect against *C. albicans* with numbers decreasing from an initial inoculum of 4.32 log cfu ml<sup>-1</sup> to 3.15 log cfu ml<sup>-1</sup> at 24 hours. There was significant inhibition of growth after 1 hour ( $p < 0.001$ ) compared to control tubes.

Combinations of oil of fennel and paraben were more effective at inhibiting and killing *C. albicans* compared to the each of the antimicrobial agent used alone. Combinations of 0.2% oil of fennel and 0.1% methyl, ethyl or propyl parabens were cidal against *C. albicans*, significantly reducing numbers of *C. albicans* after 1 hour ( $p < 0.01$ ). All of the combinations tested reduced numbers of *C. albicans* to below detectable levels. Such killing took longer with the combination containing methyl parabens (24 hours) than either the combination containing ethyl parabens (4 hours) or propyl parabens (1 hour). 0.2% oil of fennel and 0.1% propyl parabens was the most effective

**Table 4.7:** Antimicrobial action of oil of fennel and paraben combinations in Sabouraud broth against *C. albicans* over 24 hours.

Time	0 hour	1 hour	2 hour	4 hour	6 hour	8 hour	24 hour
Control	4.41±0.047	4.37±0.035	4.36±0.035	4.51±0.036	4.77±0.047	5.11±0.045	7.20±0.030
OF	4.28±0.024 <sup>∂</sup>	4.22±0.012 <sup>∂</sup>	4.18±0.050 <sup>*</sup>	3.86±0.274	3.45±0.187 <sup>*</sup>	3.49±0.165	2.53±0.048 <sup>ξ</sup>
MP	4.41±0.039	4.31±0.039	4.29±0.050	4.28±0.038 <sup>∂</sup>	4.35±0.014 <sup>ξ</sup>	4.25±0.017 <sup>ξ</sup>	4.54±0.127 <sup>ξ</sup>
EP	4.42±0.026	4.38±0.043	4.29±0.020 <sup>*</sup>	4.32±0.026 <sup>∂</sup>	4.32±0.011 <sup>ξ</sup>	4.33±0.018 <sup>ξ</sup>	4.32±0.040 <sup>ξ</sup>
PP	4.32±0.017 <sup>*</sup>	3.90±0.018 <sup>ξ</sup>	3.83±0.019 <sup>ξ</sup>	3.77±0.025 <sup>ξ</sup>	3.70±0.074	3.59±0.078 <sup>ξ</sup>	3.15±0.146 <sup>ξ</sup>
OF+MP	4.36±0.022	4.06±0.031 <sup>∂</sup>	4.01±0.042 <sup>ξ</sup>	3.55±0.054 <sup>ξ</sup>	3.14±0.068 <sup>ξ</sup>	2.82±0.125 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>
OF+EP	4.29±0.033 <sup>*</sup>	<1.93±0.22 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>
OF+PP	4.37±0.043	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>



Synergism present.

Synergy undetected due to relatively strong inhibition of microbe by one component of the combination at this time.

OF: Oil of fennel (0.2%); MP: methyl parabens (0.1%); EP: ethyl parabens (0.1%); PP: propyl parabens (0.1%). \*:  $p > 0.05$  (Significance compared to the control). <sup>∂</sup>:  $p > 0.01$  (Significance compared to the control). <sup>ξ</sup>:  $p > 0.001$  (Significance compared to the control). Each figure represents the mean of at least three independent experiments in log cfu ml<sup>-1</sup> with its SEM.



combination tested.



Combinations of 0.2% oil of fennel and 0.1% ethyl or propyl parabens were found to be synergistic over an 8 hour period against *C. albicans*. Although all of the combinations tested may have been synergistically antimicrobial at 24 hours, this could not be determined due to the relative strength of one component at this time point.

#### 4.3.4.2 *E. coli*

In the absence of antimicrobial agents, *E. coli* numbers increased rapidly to from 5.40 log cfu ml<sup>-1</sup> initially to 8.33 log cfu ml<sup>-1</sup> at 24 hours (Table 4.8). Antimicrobial regimes containing either 0.2% oil of fennel and/or 0.1% methyl, ethyl or propyl parabens exhibited a variety of bactericidal and inhibitory effects against *E. coli*. 0.2% oil of fennel had a bactericidal effect against *E. coli* for 8 hours, reducing numbers from an initial inoculum of 4.38 log cfu ml<sup>-1</sup> to 3.84 log cfu ml<sup>-1</sup>. This effect was rapid with numbers being significantly lower than the inoculum ( $p < 0.001$ ) and the growth in the control tube ( $p < 0.001$ ) after 1 hour. However, after 8 hours numbers of *E. coli* increased and there were 6.19 log cfu ml<sup>-1</sup> at 24 hours. Methyl parabens was bacteristatic with numbers not changing significantly over the duration of the experiment ( $p < 0.05$ ) and was significantly inhibitory after 6 hours. In contrast, 0.1% ethyl parabens and propyl parabens were both bactericidal against *E. coli*. 0.1% ethyl parabens reduced the initial inoculum of 5.42 log cfu ml<sup>-1</sup> by 1.59 log cfu ml<sup>-1</sup> over the first 8 hours and although there was a small recovery in *E. coli* numbers between 8 and 24 hours this was found not to be significant. Significant inhibition ( $p < 0.01$ ) of *E. coli* by ethyl parabens did not occur until 4 hours. 0.1% propyl parabens was the most inhibitory and bactericidal paraben derivative, reducing numbers of *E. coli* to below detectable levels in 2 hours. Propyl parabens significantly inhibited the organism, compared to the control, by 1 hour ( $p < 0.01$ ).

**Table 4.8:** Antimicrobial action of oil of fennel and paraben combinations in nutrient broth against *E. coli* over 24 hours.

Time	0 hour	1 hour	2 hour	4 hour	6 hour	8 hour	24 hour
Control	5.40±0.028	5.32±0.032	5.37±0.055	5.69±0.113	6.12±0.107	6.33±0.052	8.33±0.031
OF	5.38±0.043	3.95±0.056 <sup>ξ</sup>	3.71±0.033 <sup>ξ</sup>	3.60±0.017 <sup>ξ</sup>	3.70±0.083 <sup>ξ</sup>	3.84±0.036 <sup>ξ</sup>	6.19±0.049 <sup>ξ</sup>
MP	5.44±0.007	5.46±0.048	5.47±0.031	5.50±0.028	5.54±0.036 <sup>∂</sup>	5.56±0.064 <sup>ξ</sup>	5.84±0.054 <sup>ξ</sup>
EP	5.42±0.012	5.37±0.036	5.32±0.010	5.02±0.069 <sup>∂</sup>	4.60±0.138 <sup>ξ</sup>	3.83±0.286 <sup>ξ</sup>	3.90±0.052 <sup>ξ</sup>
PP	5.42±0.012	1.34±0.195 <sup>∂</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>
OF+MP	5.42±0.012	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>
OF+EP	5.42±0.012	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>
OF+PP	5.42±0.012	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>

 Synergism present.
 Synergy undetected due to relatively strong inhibition of microbe by one component of the combination at this time.

Control: Contains no antimicrobial agents; OF: Oil of fennel (0.2%); MP: methyl parabens (0.1%); EP: ethyl parabens (0.1%); PP: propyl parabens (0.1%). \*: p>0.05 (Significance compared to the control). ∂: p>0.01 (Significance compared to the control). ξ: p>0.001 (Significance compared to the control). Each figure represents the mean of at least three independent experiments in log cfu ml<sup>-1</sup> with its SEM.

Combinations of oil of fennel and paraben were more effective at inhibiting and killing *E. coli* than oil of fennel or methyl or ethyl parabens used singly. However, there was little detectable difference between the antimicrobial efficacy of propyl parabens and combination therapies. All the combinations reduced numbers of *E. coli* to below detectable levels by 1 hour and this was highly significant inhibition of bacterial numbers compared to the control ( $p < 0.001$ ).

0.2% oil of fennel and 0.1% methyl parabens was found to be synergistically active against *E. coli* for the whole of the experiment, while 0.2% oil of fennel and 0.1% ethyl parabens was synergistic for the first 4 hours. With the combination containing propyl parabens, synergy could not be demonstrated with the experimental concentrations used.

#### 4.3.4.3 *K. aerogenes*

*K. aerogenes* grew in nutrient broth without any antimicrobial agents, increasing from a concentration of 5.51 log cfu ml<sup>-1</sup> to 8.70 log cfu ml<sup>-1</sup> in 24 hours (Table 4.9). Numbers of *K. aerogenes* in tubes containing nutrient broth supplemented with 0.2% oil of fennel were significantly less than in the control tube after 1 hour ( $p < 0.001$ ). This concentration of oil of fennel was bactericidal, reducing numbers of *K. aerogenes* from 5.49 to 2.50 log cfu ml<sup>-1</sup> in 1 hour and to below detectable levels in 2 hours. Similarly, 0.1% propyl parabens significantly reduced numbers after 1 hour ( $p < 0.01$ ) reducing the numbers of *K. aerogenes* from 5.47 to 1.32 log cfu ml<sup>-1</sup> in 1 hour and to below detectable levels after 2 hours. In contrast, 0.1% methyl parabens had a bacteristatic effect against *K. aerogenes* with numbers not changing significantly during the experiment. This concentration of methyl parabens had a significant inhibitory effect compared to the control after 2 hours ( $p < 0.05$ ). 0.1% ethyl parabens had an bactericidal effect on *K. aerogenes* but did not reduce bacterial numbers below detectable levels. However, there was no significant reduction in numbers until the 24 hour measurement.

**Table 4.9:** Antimicrobial action of oil of fennel and paraben combinations in nutrient broth against *K. aerogenes* over 24 hours.

Time	0 hour	1 hour	2 hour	4 hour	6 hour	8 hour	24 hour
Control	5.51±0.064	5.51±0.058	5.60±0.134	6.09±0.054	6.64±0.070	6.96±0.161	8.70±0.055
OF	5.49±0.003	2.50±0.047 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>
MP	5.47±0.111	5.35±0.066	5.31±0.056 <sup>*</sup>	5.24±0.031 <sup>ξ</sup>	5.20±0.019 <sup>ξ</sup>	5.21±0.061 <sup>ξ</sup>	5.31±0.064 <sup>ξ</sup>
EP	5.39±0.101	5.42±0.129	5.41±0.116	5.32±0.102 <sup>∂</sup>	5.27±0.115 <sup>∂</sup>	5.22±0.137 <sup>ξ</sup>	3.99±0.203 <sup>ξ</sup>
PP	5.47±0.111	1.32±0.258 <sup>∂</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>
OF+MP	5.40±0.073	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>
OF+EP	5.42±0.104	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>
OF+PP	5.44±0.108	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>



Synergism present.

Synergy undetected due to relatively strong inhibition of microbe by one component of the combination at this time.

Control: No antimicrobial agents present; OF: Oil of fennel (0.2%); MP: methyl parabens (0.1%); EP: ethyl parabens (0.1%); PP: propyl parabens (0.1%). \*: p> 0.05 (Significance compared to the control); ∂: p>0.01 (Significance compared to the control); ξ:p>0.001 (Significance compared to the control). Each figure represents the mean of at least three independent experiments in log cfu ml<sup>-1</sup> with its SEM.



All the combinations of oil of fennel and parabens were more effective than any of the antimicrobial agents used singly. All the combinations were bactericidal and had reduced bacterial numbers to undetectable levels at the 1 hour interval. This was a highly significant reduction compared to numbers in the control tube ( $p < 0.001$ ). Synergy was seen between combinations of 0.2% oil of fennel and 0.1% methyl parabens and 0.2% oil of fennel and 0.1% ethyl parabens at 1 hour.

#### 4.3.4.4 *S. aureus*

Growth of *S. aureus* in control tubes containing tryptone soya broth without the addition of antimicrobial agents was rapid with numbers increasing from an initial inoculum of  $5.76 \log \text{cfu ml}^{-1}$  to  $8.42 \log \text{cfu ml}^{-1}$  at 24 hours (Table 4.10). 0.2% oil of fennel was bacteristatic to *S. aureus* over 24 hours. There was significant growth of *S. aureus* in the presence of 0.1% methyl parabens after 1 hour incubation at  $25^{\circ}\text{C}$  and no significant inhibition ( $p < 0.05$ ) by this concentration until the 24 hour interval. 0.1% ethyl parabens was bacteristatic against *S. aureus*, with no significant inhibition of growth compared to the control after 8 hours. In contrast to oil of fennel and methyl and ethyl parabens, a concentration of 0.1% propyl parabens had significantly bactericidal effect on *S. aureus* after 2 hours ( $p < 0.05$ ) of experimental conditions. At the end of the experiment, numbers of *S. aureus* had been reduced from an initial inoculum of  $5.76 \log \text{cfu ml}^{-1}$  to  $4.28 \log \text{cfu ml}^{-1}$ .

The combinations of oil of fennel and paraben were all bactericidal, although the combination of 0.2% oil of fennel and 0.1% methyl parabens was less effective than the most potent antimicrobial agent used alone (propyl parabens). 0.2% oil of fennel and 0.1% methyl parabens had a significant bactericidal effect after 8 hours ( $p < 0.05$ ) and reduced numbers of *S. aureus* from an initial inoculum of  $5.76 \log \text{cfu ml}^{-1}$  to  $4.91 \log \text{cfu ml}^{-1}$  at 24 hours. 0.2% oil of fennel in combination with 0.1% ethyl parabens had a significant bactericidal effect against *S. aureus* after 1 hour ( $p < 0.05$ ), reducing bacterial concentrations from  $5.76 \log \text{cfu ml}^{-1}$  (0 hour) to  $2.49 \log \text{cfu ml}^{-1}$  (24

**Table 4.10:** Antimicrobial action of oil of fennel and paraben combinations in tryptone soya broth against *S. aureus* over 24 hours

Time	0 hour	1 hour	2 hour	4 hour	6 hour	8 hour	24 hour
Control	5.76±0.042	5.93±0.090	5.97±0.122	6.10±0.116	6.40±0.141	6.81±0.158	8.42±0.052
OF	5.76±0.042	5.87±0.103	5.92±0.093	5.68±0.071	5.93±0.075 *	5.95±0.080 *	5.76±0.263 *
MP	5.76±0.042	6.00±0.040	5.92±0.059	5.97±0.031	6.06±0.064	6.31±0.085 *	7.29±0.118 <sup>∂</sup>
EP	5.76±0.042	5.92±0.065	5.85±0.094	5.85±0.064	5.89±0.055 *	5.98±0.095 *	6.07±0.109 <sup>ξ</sup>
PP	5.76±0.042	5.69±0.025	5.59±0.014	5.52±0.052 *	5.49±0.08	5.43±0.085 <sup>∂</sup>	4.28±0.503 *
OF+MP	5.76±0.042	5.75±0.019	5.65±0.019	5.63±0.012 *	5.53±0.079 <sup>∂</sup>	5.54±0.017 <sup>∂</sup>	4.91±0.037 <sup>ξ</sup>
OF+EP	5.76±0.042	4.74±0.172 <sup>∂</sup>	4.31±0.151 <sup>∂</sup>	3.61±0.233 <sup>∂</sup>	3.00±2.76 <sup>∂</sup>	3.11±0.378 <sup>∂</sup>	2.49±0.391 <sup>∂</sup>
OF+PP	5.76±0.042	3.29±0.203 <sup>∂</sup>	1.83±0.380 <sup>∂</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>	<1.00±0 <sup>ξ</sup>



Synergism present.

Synergy undetected due to relatively strong inhibition of microbe by one component of the combination at this time.

Control: No antimicrobial agents present; OF: Oil of fennel (0.2%); MP: methyl parabens (0.1%); EP: ethyl parabens (0.1%); PP: propyl parabens (0.1%). \*: p>0.05 (Significance compared to the control); ∂: p>0.01 (Significance compared to the control); ξ:p>0.001 (Significance compared to the control). Each figure in the table represents the mean of at least three independent experiments in log cfu ml<sup>-1</sup> with its SEM.

hours). The combination of oil of fennel with propyl parabens was the most effective antimicrobial preparation. A significant bactericidal effect ( $p < 0.01$ ) was measured after 1 hour with a reduction of  $2.47 \log \text{cfu ml}^{-1}$ . However, at the 4 hour interval this combination had reduced numbers of *S. aureus* to below detectable levels ( $< 1.00 \log \text{cfu ml}^{-1}$ ).

Synergy was observed between oil of fennel and all three alkyl derivatives of parabens. Combinations containing ethyl parabens and propyl parabens were synergistic for all of the experimental measurements, while synergy between methyl parabens and oil of fennel was only detected at the 1-6 hour time intervals.

#### **4.4 DISCUSSION**

Many studies which evaluate novel antimicrobial agents employ permutations of the broth dilution technique (Miles and Amyes, 1996), which determines MICs for single agents, and the Checkerboard Technique (Eliopoulous and Moellering 1991), which evaluates combinations of antimicrobial agents. However, these techniques have been designed for evaluation of hydrophilic antimicrobials in laboratory media where visual or spectrophotometric measurements of microbial growth can be made. Therefore, for the purposes of this study, these procedures were modified to take into account the hydrophobic nature of oil of fennel and the opacity of Osmolite.

The Broth Dilution Technique was used to measure MIC and MBC of oil of fennel and parabens in laboratory media and Osmolite. It also forms the basis of the Checkerboard Technique. In most published studies 100µl or 1000µl of test media have been used in the assays. This has the advantage of minimizing the quantity of media used in assays, especially when large numbers of permutations are evaluated. However, such volume sizes have high volume to surface ratios which can increase the opportunity for volatile antimicrobial agents such as oil of fennel to evaporate. In this study 10 ml of growth media was used which not only reduced the surface to volume ratio but also facilitated the formation of a stable suspension of oil of fennel in the growth medium after vortex mixing. Formation of a fine and stable suspension of oil of fennel droplets in the growth media was found to be very important because it had a profound effect on the reproducibility of results. Insufficient vortex mixing produced large droplet sizes which decreased the surface area of oil of fennel in contact with the growing microorganisms. Large droplet sizes also increased the likelihood of the oil of fennel coalescing at the surface of the growth media during incubation. This increased the opportunity for evaporation of the essential oil and reduced contact with the microorganisms in the growth media giving a false result.



Other studies have tried to overcome the problems of poor solubility by adding detergents into the growth media. Dimethylsulphoxide (Hili *et al*, 1997), Polysorbatum 80 (Janssen *et al*, 1986) and Tween 80 (Juven *et al*, 1994; Carson *et al*, 1995; Carson and Riley, 1995) are examples of some of the dispersal agents used to solubilize essential oils. However, Hili *et al*, 1997 found that the activity of cinnamon oil against three bacteria and four yeasts decreased fifty-fold when dimethylsulphoxide was used as a dispersal agent. Juven *et al*, (1994) found a similar neutralizing action with Tween 80 when used in assays evaluating the antimicrobial properties of thyme oil and its main constituent thymol. Therefore, the addition of such agents to augment the solubility of the oil of fennel into the aqueous environment was rejected in this study.

Further adjustments also had to be made to the Broth Dilution Technique to address the difficulty of making normal visual growth determinations in laboratory media compared to Osmolite. In cases where Osmolite was used as the growth media, bacteria were enumerated after a  $10^2$  dilution in 0.01M PBS, this enabled an approximate comparison with the original inoculum of microorganisms added to the growth medium before incubation. This yielded similar, if not slightly more accurate, information about growth in the presence of the antimicrobial agent than visual assessment. However, it had the disadvantage of considerably increasing the workload and consumption of materials and so was reserved for experiments where visual assessment could not be made.

The Checkerboard Technique, although widely used, is the subject of some controversy. Although the rationale is easy to understand and the mathematics required to interpret the results are simple, there are several disadvantages (Eliopoulous and Moellering, 1991). Firstly, Checkerboard Assays only yield inhibitory data if the microorganisms from each tube or well are not enumerated. Secondly, Fractional Inhibitory Concentrations (FIC) index calculations and

isobolograms, which are the normal means of interpreting Checkerboards assume that all antimicrobial agents have linear dose-effect responses. Thirdly, Checkerboards as they are usually performed provide an insufficient quality of data to characterise dose-response relationships because they are incapable of measuring graded responses of microorganism to antimicrobial agents. Finally, results are only measured at one time point, giving a static view rather than the dynamic view provided by the killing curve technique. Norden and co-workers (1979) found that Checkerboard assays had poor correlation with results from killing curve assays, which are considered to be more accurate. They recommended the standardization of criteria used to define synergy and interpret Checkerboards. More recently, Bowker *et al*, (1996) found that the technique had poor reproducibility and FIC calculations often produced results indicating synergy when antimicrobial agents were added to themselves. These authors concluded that the checkerboard Technique is not a useful method of investigating antimicrobial combinations.

The modifications, made in this study, to the Broth Dilution Technique and to how data from the Checkerboards was presented, overcame some of the inaccuracies and deficiencies of the Checkerboard Technique as usually practised. In this study each tube was sampled and so cidal and inhibitory data rather than inhibitory data alone was obtained. In addition, the data from the viable counts that replaced visual assessment of growth in assays using Osmolite increased the accuracy. A modified form of isobologram was used to represent results, this facilitated calculations determining synergy to be made directly from the results without requiring FIC calculations and qualitative assessments of the shape of isobolograms. This meant the shortcomings of the FIC calculations identified by Bowker *et al*, (1996) were avoided. In contrast, to the work of Norden *et al*, (1979) the results from the Checkerboards in this study were in agreement both with the MIC and MBC experiments and subsequent Killing Curve Assay results. The modifications made to the Broth Dilution Technique greatly increased the number of tubes in which microorganisms were

enumerated in the Checkerboard Assay but also increased the accuracy and the value of the resulting data.

It is also believed by some authors that prevention of "carryover" of antimicrobial agents from the test media into enumeration media is important to ensure the accuracy of MIC and MBC measurements and Checkerboards results. Therefore the neutralizing characteristics of detergents such as Tween 80 has also led to their use in preventing the carryover. In addition to its inhibitory action against some essential oils, Tween 80 is also known to inactivate parabens and some other antimicrobial agents (Baker *et al*, 1983; Hugo and Russell, 1992; Juven *et al*, 1994). Therefore, the first aim of this chapter was to evaluate the use of Tween 80 as an aid to recovery of microbial cells from antimicrobial assays. The results showed that there was no significant benefit in adding Tween 80 into enumeration media when evaluating the antimicrobial properties of oil of fennel. It was also considered that the carryover effect was likely to be negligible since the quantities of antimicrobial agent being carried over were very small in comparison with the volume of the enumeration media. Therefore, in experiments where microorganisms were treated with oil of fennel and parabens no Tween 80 was included in any of the enumeration media.

Other experimental conditions present in this study were determined by the properties of the materials being investigated. For example, parabens were used up to the maximum concentrations at which they were soluble in growth media and oil of fennel was used up to a concentration of 1% which was the maximum that can be tolerated organoleptically in food. All experiments were incubated at 25°C which simulated the normal temperature at which enteral nutrient solutions are used.

The second aim of this chapter was to investigate the possibility of inhibiting the growth of microorganisms in enteral nutrient solutions using oil of fennel and/or combinations of oil of fennel and parabens. The results showed that there were

considerable variations in the efficacy of oil of fennel, parabens and combinations at inhibiting different test organisms. In addition, there was also a large difference between the activities of these antimicrobial agents in laboratory media compared to Osmolite. Variation in sensitivity to preservatives used in the study was particularly apparent in laboratory media. For example, the minimum concentration of oil of fennel required to inhibit *K. aerogenes* in laboratory media was 0.008%, while a concentration of 1% oil of fennel had no detectable inhibitory effect on *P. aeruginosa* or *Ent. cloacae*. The minimum inhibitory concentrations of methyl parabens in laboratory media were in the range 0.07-0.15%, while ethyl and propyl parabens were effective at concentrations in the range 0.05-0.125% and 0.03-0.075% respectively. This finding shows an increase in the activity of parabens with increasing alkyl chain length and is in agreement with the findings of Oka (1960). The variation in the range of minimum inhibitory concentrations was less evident in Osmolite than laboratory media. The range of MICs of methyl parabens was 0.15->0.3%, while the range of MICs for oil of fennel in Osmolite was even less (0.9->1%). This is likely to be because of the overall decrease in antimicrobial activity of oil of fennel and parabens when used in Osmolite.

The minimum bactericidal concentrations followed a similar pattern to the minimum inhibitory concentrations, except that all the MBCs were above the maximum concentrations of preservatives tested in Osmolite. The minimum bactericidal concentrations of oil of fennel were in the range 0.015->1%. Three of the microbial strains tested had a MBC which exceeded the maximum concentration of oil of fennel used in experiments. The ranges of minimum bactericidal concentrations of methyl, ethyl and propyl parabens in laboratory media were 0.25->0.3%, 0.15->0.3% and 0.07->0.2% respectively. These results also show an increase in antimicrobial activity with an increase in alkyl chain length of the parabens. In Osmolite, the activity of oil of fennel and methyl parabens was reduced so much that minimum bactericidal

concentrations were not able to be determined in all cases except when measuring the inhibition of *C. albicans* with methyl parabens.

Variation in the sensitivity of organisms to non-antibiotic antimicrobial agents is a function of their cell envelope structure (Hugo and Russell, 1992). In general, Gram-negative organisms are more resistant to antimicrobial agents than Gram-positive organisms and fungi (Hugo and Russell, 1992). This is as a result of their more complex cell wall structure. However, the results of this study showed no relationship between Gram-stain reaction and sensitivity to oil of fennel and paraben. This is possibly due to a combination of the statistically small number of microbial strains involved in the study and each strain's individual resistance to oil of fennel and paraben. However, some studies, such as that of Deans and Richie (1987) who investigated the antimicrobial activity of 50 essential oils against 25 genera of microorganisms, have also found no relationship between sensitivity to essential oils and Gram-stain reaction.

*P. aeruginosa* was the most resistant organism to oil of fennel, parabens and combinations. This is not surprising because Deans and Richie (1987) found that oil of fennel actually stimulated the growth of their strain of *P. aeruginosa*. High concentrations of paraben are also required to inhibit the growth of *P. aeruginosa* (Davidson, 1993). This high resistance may be explained by the ability of some Pseudomonads and particularly *P. aeruginosa* to produce enzymes which breakdown phenolics, such as those present in oil of fennel, and parabens (Hugo, 1991b citing Davey, 1961; Beveridge and Hugo, 1964a,b; Beveridge and Hart, 1970). However, it is more likely that the large amount of O-polysaccharide present in this strain of *P. aeruginosa* as determined in Chapter Five, and the extra phosphates groups present in the Lipid A of Pseudomonal LPS (Proctor *et al*, 1995) are responsible for the extra resistance of this organism. These structures make the cell envelope a hydrophilic

barrier which is highly effective at preventing hydrophobic antimicrobial agents, such as oil of fennel and parabens, from disrupting the cytoplasmic membrane.

*K. aerogenes* was the most sensitive microorganism to oil of fennel. This was an interesting result because this strain of *Klebsiella* has a large glycocalyx-type capsule. Such capsules are composed from sugars such as uronic acid which are hydrophilic. A capsule is therefore, another structure produced by an organism that has the potential to prevent access of hydrophobic antimicrobial agents to the cytoplasmic membrane. Although vortex mixing may have removed the capsule leaving cells more vulnerable to oil of fennel, a similar effect was not observed with cells treated with parabens where *K. aerogenes* was no more sensitive than other Gram-negative bacterial species used in experiments. This may indicate that oil of fennel interacts with the capsule in such a manner that it is released from the microbial cell. This could be investigated by electron microscopy of treated microorganisms.

In this study there was a large difference between the activity of oil of fennel, parabens and combinations when used to inhibit organisms growing in laboratory media compared to Osmolite. This is not an unexpected result. Several studies have shown that the activity of phenolic antimicrobial agents when used in foods is considerably reduced (Shelef *et al*, 1984; Juven *et al*, 1994). Several hypotheses have been put forward to explain such results but it is likely that a combination of all or more than one is an explanation.

Firstly, phenolic compounds such as parabens and some of the most significant components of oil of fennel readily interact with the amino groups of proteins. These interactions depend on the characteristics of the protein, the pH of the medium and characteristics of the phenolic molecule such as size, conformational flexibility and solubility in the aqueous phase (Juven *et al*, 1994). Complexes are formed as a result of hydrogen bonds and hydrophobic interactions between the peptide bonds of



the protein and the phenolic molecule (Juven 1994 citing Synge, 1978; Haslam and Lilley, 1988; Spencer *et al*, 1988). It is now widely accepted that the protein content of the food will have a deleterious effect on the antimicrobial activity of essential oils (Shelef, 1983; Nychas, 1995). Juven *et al*, (1994) found that the effectiveness of thymol, a phenolic constituent of thyme oil, was greatly reduced in growth media supplemented with protein (Bovine serum albumen: BSA) compared to either nutrient agar (5mg ml<sup>-1</sup> of amino acids) or a media containing no organic nitrogen sources. Anethole is the most abundant constituent in oil of fennel (Max, 1992; Tisserand and Balacs, 1995) and is considered to be the most important constituent with antimicrobial properties (Karapinar and Aktug, 1987). The large drop in the antimicrobial activity observed when organisms were cultured in Osmolite may partly be explained by its high protein content (4 g per 100 ml) interacting with anethole and other phenolic constituents.

Secondly, the hydrophobic nature of parabens or components of oil of fennel will cause them to preferentially dissolve in the lipid phase of foods (Hugo and Russell, 1992). This will prevent them from having contact with organisms proliferating in the aqueous phase of a food system. Osmolite is a milk-based enteral nutrient solution and is an emulsified combination of milk-proteins and vegetable oils (3.4 g per 100 ml) such as sunflower, canola and fractionated coconut. Therefore, the composition of Osmolite is such, that the majority of parabens or oil of fennel may be unavailable to interact with microorganisms.

Thirdly, microorganisms may be protected from antimicrobial agents by an accumulation of hydrophilic proteins and carbohydrates on the surface of microbial cells. Caseinates and maltodextrins present in Osmolite may form loose hydrogen-bond mediated or non-specific aggregates around microbial cells that resist access by hydrophobic antimicrobial molecules to target structures on the microbial cell.

Finally, the phenotypic expression of many of the microbial structures that are important in determining resistance to antimicrobial agents, are dependent on the growth environment. This is known to be true, not only for Gram-negative organisms but also for Gram-positive bacteria and yeasts (Kroll, 1990). Many studies of Gram-negative bacteria, including the results presented in Chapter Five, have demonstrated that the expression of O-polysaccharide is particularly dependent on the growth conditions (Kelly *et al*, 1989; Nelson *et al*, 1991; Allan and Poxton, 1994). Variability in the presence and length of O-polysaccharide has an effect on the ability of antimicrobial agents (particularly hydrophobic compounds) to access microbial targets on the cytoplasmic membrane and in the cytoplasm itself (Proctor *et al*, 1995). In Chapter Five, the LPS was shown to be different in *E. coli* or *P. aeruginosa* cultured in laboratory media compared to when they were cultured in Osmolite. It is possible that these increases in the heterogeneity of O-polysaccharide chain size were partly responsible for the increase in the resistance of these organisms to oil of fennel and methyl parabens in Osmolite. In addition there might have been alterations in the phenotypic expression of structures such as capsules, glycocalyxes and alginate (present on both Gram-negative and Gram-positive bacteria), which convey resistance to organisms. In Gram-positive organisms the composition of lipids in the cytoplasm are known to be both a factor dependent on the growth environment and a factor affecting resistance to antimicrobial agents (Hugo and Russell, 1992). Changes in these additional factors may explain the greater resistance of Gram-positive organisms growing in Osmolite.

Combinations of preservatives have advantages over the use of single agents in that they are less likely to encounter microbial resistance and offer the possibility of synergism (Eliopoulous and Moellering, 1991). This study focused on combinations between oil of fennel and the methyl derivative of parabens to reduce the vast number of permutations that would have been generated by evaluating other types of parabens using the Checkerboard Technique. However, in killing curve assays it was possible to



expand the investigation to include combinations of oil of fennel with ethyl and propyl parabens. The results of checkerboard and killing curve assays showed that oil of fennel and methyl parabens formed both combinations which had an additive antimicrobial effect and combinations which were synergistically antimicrobial.

Results of Checkerboard assays indicated a clear relationship between the antimicrobial activity of the individual components of combinations and the activity of the combination itself. The variation in the activity of combinations against different microbial strains and in different growth media followed the same patterns as the individual components. For example, *K. aerogenes* was the most sensitive microorganism to combinations of oil of fennel and methyl parabens in laboratory media, while *P. aeruginosa* and *Ent. cloacae* were the most resistant strains. In Osmolite, the variation in resistance to combinations of oil of fennel and methyl parabens was less than in laboratory media. *C. albicans* was the most sensitive to combinations of oil of fennel and methyl parabens (0.45% oil of fennel, 0.05% methyl parabens), while *P. aeruginosa* was the most resistant strain (0.5% oil of fennel, 0.4 methyl paraben).

Similarly, results from the killing curve assays, which gave a dynamic view of microbial growth in the presence of combinations and enabled the detection of synergy, showed that *K. aerogenes* and *E. coli* were the most sensitive organisms to combinations of oil of fennel and parabens. Combinations of oil of fennel with methyl, ethyl and propyl parabens were very powerful against the microbes tested, in most cases reducing numbers of *C. albicans*, *E. coli* and *K. aerogenes* to below 1.00 log cfu ml<sup>-1</sup> in 1 hour. However, these combinations of oil of fennel and parabens were less effective against *S. aureus*. A combination of 0.25% oil of fennel and 0.1% methyl parabens was bacteristatic to *S. aureus*, while the combination containing ethyl parabens reduced numbers to 2.49 log cfu ml<sup>-1</sup> over 24 hours. The most effective combination against *S. aureus* was oil of fennel and propyl parabens reduced numbers

to below  $1.00 \log \text{cfu ml}^{-1}$  in 4 hours. These results indicate alkyl chain length of the parabens is important in determining the activity of the combination as well as the individual paraben. They also show that ethyl and propyl parabens, rather than methyl parabens, may have a greater potential as part of preservative combinations for inhibiting growth of microbial contaminants in enteral nutrient solutions. This should be investigated by further experiments in milk-based enteral nutrient solutions. In addition, it would also be interesting to evaluate the activity of combinations containing ethyl and propyl parabens against *Ent. cloacae* and *P. aeruginosa*.

The third and final aim of this chapter was to determine the extent of synergy between oil of fennel and paraben. Results from the Checkerboard assays demonstrated that oil of fennel and methyl paraben combinations were synergistically active against all microbes in laboratory media and 4/6 strains in Osmolite. Furthermore, Killing Curve assays showed that combinations of oil of fennel with ethyl parabens were not only synergistically active, in laboratory media, against *C. albicans*, *E. coli*, *K. aerogenes* and *S. aureus* but they had greater activity than combinations containing methyl parabens.

In cases, where synergy was not determined by these techniques, it is difficult to predict whether combinations were truly not synergistic or whether it was function of the concentrations of reagents used in the experiments. For example, the Checkerboard results showed that there was no synergy between oil of fennel and methyl parabens against *C. albicans* or *S. aureus* when these organisms were growing in Osmolite. However, these results were marginal and it is possible that slightly higher concentrations of each antimicrobial would have yielded a positive result for synergy. Similarly, it is clear from the Killing Curve results that, although synergy was not found for the entire 24 hours of most experiments, if the concentrations of parabens had been lower in the experiments, then synergy would have been detected. These findings indicate that synergy between oil of fennel and paraben is only present

in a restricted range of concentration and these ranges of concentrations vary radically depending on the growth medium. It also demonstrates the difficulties of determining the presence of synergy which exists at a restricted range of concentrations using techniques such as the Checkerboard technique which covers a wide range of concentrations and is thus, relatively insensitive. Hugo and Russell (1992) believe there to be relatively few combinations of preservatives which are synergistic. These results indicate that the techniques available are not always very effective at locating the concentrations at which synergy exist and thus there may be many more synergistic combinations available to the microbiologist than currently documented in the literature.

The mechanism for the synergy described in this study is uncertain. Although there are several mechanisms for synergy between antibiotics, only permeabilization synergy has been applied to preservatives (Denyer *et al*, 1985). This is where one component of the combination disrupts the microbial cell wall and cytoplasmic membrane to enable access of the other (or both) to the cell interior. Parabens are known to form synergistic antimicrobial combinations with phenolic compounds (2-phenylethanol and 2-phenoxyethanol) (Denyer *et al*, 1985 citing Richards and McBride, 1971; Wilkinson, 1975; and Boehm, 1968). It is possible that parabens forms a synergistic relationship with phenolic constituents in oil of fennel, such as anethole. The mode of action of parabens and the phenolic compounds such as those present in oil of fennel are thought to be similar (Nychas, 1995). Studies have shown that phenolics can cause membrane dysfunction as well as membrane disruption, resulting in decreases in nutrient uptake, nucleic acid synthesis and ATPase activity (Eklund, 1980; Degre and Sylvestre, 1983; Degre *et al*, 1983; Rico-Munoz *et al*, 1987; Ruiz-Barba *et al*, 1990; Denyer and Hugo, 1991; Hugo 1991a; Kabara and Eklund, 1991; Eklund and Nes, 1991; and Martin, 1992). Eklund (1980) and Freese *et al* (1973) cited by Davidson (1993) stated that Gram-negative bacteria were most likely to be resistant to parabens owing to a screening effect by the cell wall lipopolysaccharide layer. Therefore it is

possible that phenolic compounds or other components in oil of fennel cause permeabilization of the cell wall and disruption of the cell membrane, which increases the access for parabens.

The experiments in this study showed that most of the effective combinations in enteral nutrient solutions contained concentrations of methyl paraben that were higher than the legal limit in the UK. An alternative approach to preservation of enteral nutrient solutions, which could reduce risk to patients, is to inhibit the harmful products produced by contaminating microorganism and reduce the virulence of organisms. However, this is only possible if initial amounts of contaminating microorganisms are low. Sub-lethal concentrations of antibiotics have been shown to damage or change organisms in such a manner that their virulence is reduced (Lorian and Gemmell, 1991; Nelson *et al*, 1991). However, it is not known whether sub-lethal concentrations of preservatives might have the same effect.

#### **4.5 SUMMARY.**

This chapter has shown that combinations of oil of fennel and methyl parabens can be successfully used to inhibit the growth of microorganisms that commonly contaminate enteral nutrient solutions, in laboratory media and Osmolite. Although in most cases the concentration of methyl parabens used in combinations was in excess of the legal limit, parabens with higher alkyl chain length could be used to replace methyl parabens and increase efficacy. The results also show that oil of fennel forms synergistic antimicrobial combinations with methyl, ethyl and propyl parabens against most organisms, if the correct concentrations of antimicrobial agents are identified. Further research is required to ascertain whether sub-lethal combinations of oil of fennel and parabens can be successfully used to decrease the risk of microbial contamination to enterally fed patients by inhibiting synthesis of harmful microbial products or decreasing microbial virulence.

## CHAPTER 5

### CHANGES IN THE PATHOGENICITY OF MICROORGANISMS GROWING IN ENTERAL NUTRIENT SOLUTIONS IN THE PRESENCE OR ABSENCE OF NOVEL PRESERVATIVES

#### 5.1 INTRODUCTION

Opportunistic pathogenic Gram-negative bacteria are the most commonly isolated contaminants of enteral nutrient solutions (Navajas *et al*, 1992). These are microbes of relatively low pathogenicity (Smith, 1995) which cause infections in immunocompromised patients such as the enterally fed (Anderton, 1993). They are also frequently cited in the literature as the cause of nosocomial infections associated with contaminated enteral feedings (Anderton, 1993).

Smith, (1995) is of the opinion that research into the pathogenicity of such microbes should take a different approach to the study of other microorganisms. This is because opportunistic pathogens are often introduced directly into host tissues so investigation of penetration and survival of mucous surfaces is not relevant and interference with host defences has a low priority because host defences are already substantially reduced. This author considers the main research priority for these groups of pathogens to be the investigation of the mechanisms by which these microbes damage the host and multiply *in-vivo*. However, the priorities for research into the pathogenicity of microorganisms which contaminate enteral nutrient solutions are less clear. This is because the precise mechanisms by which contaminating microorganisms cause disease in the enterally fed patient are still a matter of speculation. Bacteraemia, septicaemia and respiratory infections represent the most important complications associated with the consumption of contaminated enteral nutrient solutions. When these conditions are caused by opportunistic pathogens, it is typically the responses of the host's own immune system that cause more damage than the direct effect of microbial toxins on the host cells

(Smith, 1995). One bacterial structure that is an important stimulus of such immunopathological reactions is lipopolysaccharide. The structure and biological activity of this molecule has been discussed in Section 1.6.3.1.

Nutrient availability in the growth environment has been shown to have a profound effect on the distribution of O-polysaccharide chain lengths of LPS molecules on the surface of Gram-negative bacteria (Weiss *et al*, 1986; Dodds *et al*, 1987; Kelly *et al*, 1989) and the ratio of core and O-polysaccharide expressed on the cell surface (Nelson *et al*, 1991). High concentrations of NaCl, MgCl<sub>2</sub>, glycerol and sucrose or low concentrations of phosphates and low pH have been found to increase the production of long chain lipopolysaccharide (McGroarty and Rivera, 1990). Growth of *E. coli* in heat-inactivated sheep serum or magnesium depleted conditions caused an increase in expression of LPS core and decreased production of O-polysaccharide (Nelson *et al*, 1991). In the nitrogen deficient/high carbon conditions, chosen by these authors to enhance capsule production, an increase in production of O-polysaccharide was observed. Such changes in the phenotypic expression of O-antigen have been shown to affect bacterial pathogenicity by altering resistance to serum (Allan and Poxton, 1994) and phagocytosis (Williams *et al*, 1983; Weiss *et al*, 1986). However, it is also possible that other aspects of microbial pathogenicity, in which O-polysaccharide is important, may also be affected.

Some bacteria generate more than one chemotype of lipopolysaccharide. For example, *P. aeruginosa* is known to produce two types of chemically distinct LPS: the common antigen or "A-bands" and the "B-bands" which define the serological type (Rivera *et al*, 1988). A-band LPS has a chemically distinct lipid A-core region and an O-polysaccharide composed of short chains of repeating units containing three D-rhamnose or D-glucose residues (Rivera and McGroarty, 1989). A-band LPS is common amongst many strains of *P. aeruginosa* and is the major antigen in non-typeable clinical strains (Lam *et al*, 1989). B-band LPS has longer chain O-polysaccharide, composed of repeating units of amino sugars (McGroarty and Rivera, 1990). In these circumstances nutrient availability



in the growth media can affect the proportions of each type of LPS being produced (McGroarty and Rivera, 1990; Makin and Beveridge, 1996). Changes in the proportions of A and B band LPS have been shown to influence the adherence of *P. aeruginosa* by altering hydrophobicity of the bacterial cell surface (Makin and Beveridge, 1996). It is also possible that such changes would alter resistance to phagocytosis which is dependent on cell surface hydrophobicity amongst other factors.

There are no reports investigating whether nutrient availability in the growth environment alters endotoxicity. This is possibly because the structure of lipid A is generally conserved. However, it is possible endotoxicity may be altered if nutrient availability affects the amount of LPS expressed on the outer membrane surface and therefore the access of lipid A to macrophage receptors (Nelson *et al*, 1991).

In addition to the effects of nutrient availability, the expression of some microbial virulence determinants has been shown to be altered by the addition of sub-lethal concentrations of antimicrobial agents (Nelson *et al*, 1993; Lorian and Gemmell, 1991). However, the potential of most preservatives to alter microbial pathogenicity remains unstudied (See Section 1.6.2.5). Therefore, it is no surprise that there is an absence of studies which investigate whether sub-lethal concentrations of oil of fennel may effect the virulence properties of microorganisms. However, one report by Hitokoto *et al*, (1980) found that anethole, a major component of oil of fennel, inhibited aflatoxin production in a number of *Aspergillus* spp. at a concentration of 1mg ml<sup>-1</sup>. Similarly, studies with parabens are limited. Robach and Pierson (1978) demonstrated that 100µg ml<sup>-1</sup> concentrations of methyl and propyl parabens inhibited toxin formation in laboratory media by *Clostridium botulinum*. Draughton and Co-workers (1982) found that 1000µg ml<sup>-1</sup> was effective at preventing toxigenesis by *Cl. botulinum* in canned comminuted pork. 200 µg ml<sup>-1</sup> of propyl parabens was sufficient to inhibit the production of a protease by *Aeromonas hydrophila* (Venugopal *et al*, 1984). Although, there are no reports as to whether parabens or oil of fennel affect the expression of other virulence



factors such as lipopolysaccharide, the possibility that these antimicrobial agents may be used to reduce microbial pathogenicity is intriguing and may be of potential use in preventing disease associated with contaminated enteral feedings.

In summary, our understanding of the pathogenicity is mainly based on studies conducted in laboratory media. Many studies have shown that nutrient limitation or supplementation of laboratory media cannot only induce changes in the expression of lipopolysaccharide (Weiss *et al*, 1986; Kelly *et al*, 1989; Nelson *et al*, 1991; Allan and Poxton, 1994) but also a wide range of other Gram-negative bacterial virulence structures. These include capsules (Mengistu *et al*, 1994), fimbriae (McGee *et al*, 1979), extracellular proteins (Sokol and Woods, 1984 ; Mouriño *et al*, 1994; Ebel *et al*, 1996), and outer membrane proteins (Darveau *et al*, 1983; Brown and Williams, 1985; Camprubi *et al*, 1992; Allan and Poxton, 1994). Therefore, it is conceivable that the pathogenicity of Gram-negative bacteria growing in enteral nutrient solutions is different to that previously documented in the literature.

The study of Gram-negative bacterial pathogenicity in enteral nutrient solutions is important because: it is difficult to ascertain whether existing information about Gram-negative pathogenicity (determined in laboratory media) is relevant when bacteria are growing in enteral formulas; information from such studies would support the validity of microbiological standards for enteral feeding systems; understanding about the potential of microorganisms growing in enteral feeds to cause infections would be enhanced; and this information may enable the development of antimicrobial agents which reduce the pathogenicity of bacteria by altering the phenotypic expression of virulence determinants. The study of lipopolysaccharide is an appropriate starting point for research in this area because much is already known about its structure and biological activity; it mediates in many aspects of Gram-negative bacterial pathogenicity; and its phenotype is dependent on growth conditions. Therefore, the aims of this chapter are as follows:

- To investigate whether the phenotypic expression of lipopolysaccharide in enteral nutrient solutions is similar to that already established by conventional means in laboratory media.
- To investigate whether aspects of Gram-negative bacterial pathogenicity mediated by lipopolysaccharide were altered when bacteria were grown in enteral nutrient solutions compared to conventional means in laboratory media.
- To investigate whether sub-inhibitory concentrations of oil of fennel, parabens and combinations of these preservatives alter the phenotypic expression of lipopolysaccharide in laboratory media and enteral nutrient solutions.

## **5.2 MATERIALS AND METHODS**

### **5.2.1 Materials**

The THP-1 human monocyte cell line and L929 murine fibroblast cell line were obtained from the European Collection of Animal Cell Culture (ECACC, Salisbury, Wiltshire UK). Bacterial strains were obtained from the sources described in Section 2.1.1 and chemicals and cell culture medium were purchased as described in Section 2.1.2.

### **5.2.2 Methods**

#### **5.2.2.1 Preparation of lipopolysaccharide using Proteinase K digestion of whole bacterial cells**

Lipopolysaccharide was prepared by Proteinase K (Sigma) digestion of whole cells using a modification of the method described by Hancock and Poxton (1988). Enteral nutrient solution and nutrient broth (20 ml) were inoculated with an overnight culture (Section 2.2.2) to give an approximate  $5.00 \log \text{cfu ml}^{-1}$  concentration of bacteria. Cultures were incubated for 24 hours at 25°C and centrifuged at  $2300 \times g$  for 15 minutes to pellet the cells. Bacteria were washed twice in 0.01M PBS, resuspended in 1.5 ml of 0.01M PBS to give an absorbency of 0.5 to 0.6 at 490 nm using a Dynatech MR 5000 (Dynatech Laboratories Limited, Daux Road, Billingshurst, West Sussex, UK). Cells were pelleted at  $10,000 \times g$  for 10 min in a microcentrifuge, resuspended in 50µl SDS-PAGE buffer (0.125M Tris-HCl pH 6.8, 4% SDS, 20% Glycerol, 2% 2-mercaptoethanol, 0.004% bromophenol blue) and boiled at 100°C for 15 minutes. Each sample was incubated in a water-bath for 24 hours at 60°C with 10µl of Proteinase K ( $2.5 \text{ mg ml}^{-1}$ ). After incubation samples were used directly on SDS-PAGE gels 10µl per well.

#### **5.2.2.2 Preparation of lipopolysaccharide using hot phenol extraction**

The hot aqueous phenol method of Westphal and Luderitz (1954) as described by Hancock and Poxton (1988) was used to extract lipopolysaccharide from 1 litre batches

of selected bacteria. In all cases the final ultracentrifugation step of this method was omitted.

#### 5.2.2.2a Bacterial Cell Culture and Harvesting

2 x 500 ml of nutrient broth or enteral nutrient solutions were inoculated with 10 ml of a single overnight culture prepared in the manner described in 2.2.2 and incubated at 25°C for 24 hours. In some cases, nutrient broth and enteral nutrient solutions were supplemented with oil of fennel, methyl parabens and combinations of these preservatives at concentrations determined in Chapter Four. Bacterial cultures were harvested at 8000 x g. Cells were washed twice in 100 ml aliquots of sterile pyrogen free 0.01M PBS. Finally, the harvested cell mass was suspended in 20 ml of sterile pyrogen free 0.01M PBS, frozen at -20°C and lyophilized in a Modulyo Freeze-dryer (Edwards High Vacuum International, Crawley, Sussex, UK).

#### 5.2.2.2b Hot Aqueous phenol Extraction

A 2% w/v suspension of the lyophilized material was made in pyrogen free water and heated to 67°C for 15 minutes. This was mixed with an equal proportion of 90% w/v phenol solution and incubated for a further 15 min at 67°C. The mixture was cooled to room temperature in ice and centrifuged at 9000 x g. The aqueous phase was set aside and the phenolic phase washed twice with pyrogen free water, reserving the aqueous phase on each occasion. The aqueous phases from extractions were pooled and dialysed in boiled dialysis tubing against running water for approximately 24 hours and then in distilled water for a further 4 hours. The aqueous phase was concentrated using a rotary evaporator (Büchii, Flawil, Switzerland) until reduced to approximately 1/5 of its original volume. The concentrated extract was then frozen at -20°C and lyophilized.

### 5.2.2.3 Preparation of lipopolysaccharide samples for polyacrylamide gel electrophoresis

All lipopolysaccharide samples extracted using the method described in 5.2.2.2 were further purified using Proteinase K to remove any proteinaceous impurities either from the enteral nutrient solution or from the outer membranes of the bacteria. Thus, 2mg of extracted lipopolysaccharide was dissolved in 100 $\mu$ l of double strength SDS-PAGE buffer (0.125M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 2% mercaptoethanol, 0.002% bromophenol blue) and 100 $\mu$ l of pyrogen free water. Samples were then boiled for 15 minutes and cooled to room temperature. 50 $\mu$ l of Proteinase K (2.5 mg ml<sup>-1</sup>) was added to each sample before incubation at 60°C for 24 hours.

### 5.2.2.4 Polyacrylamide gel electrophoresis

PAGE was performed on 12% or 14% acrylamide slab gels using the discontinuous buffer system of Laemmli (1970). Gels used to analyse lipopolysaccharide were made according to the proportions shown in Table 5.1. Double strength stacking and separating buffers were used in all cases and gel solutions were degassed for 15 min before polymerisation. Polymerisation was achieved using NNN'N'-tetramethyl-1,2-diaminoethane (TEMED) and ammonium persulphate (15mg ml<sup>-1</sup>). Other solutions used were as follows:-

- **Stacking Buffer** (double strength, pH 6.8): 0.25 M Tris-HCl, 0.2% w/v SDS in de-ionised water.
- **Running Buffer** (double strength, pH 8.8): 0.75 M Tris-HCl, 0.2% w/v SDS in de-ionised water.
- **Acrylamide Stock Solution** (40% w/v): 100g acrylamide (BDH Electrophoresis Grade) and 2.7g methylene bis-acrylamide (BDH, Electrophoresis Grade) in 250ml de-ionised water.
- **Electrode Buffer** (pH 8.3): 0.303% Tris, 1.44% glycine, 0.2% SDS in de-ionised water.

**Table 5.1:** Preparation of polyacrylamide gels for analysis of lipopolysaccharide.

<b>Reagent</b>	<b>Separating gel 14% acrylamide</b>	<b>Stacking gel 4% acrylamide</b>
De-ionised water	3.45ml	3.5ml
Separating buffer	17.5ml	N/A
Stacking buffer	N/A	5.0ml
40% acrylamide stock	12.25ml	1.0ml
TEMED	50µl	20µl
Ammonium persulphate	1.75ml	0.5ml

The separating gel was poured between two clean glass plates (160 mm x 125 mm x 1.5 mm) using a needle and syringe, over-laid with water saturated butan-2-ol and allowed to polymerize. After the removal of the butan-2-ol and one wash of the gel surface with pyrogen-free water, the stacking gel was poured and a 20 well comb inserted. Following polymerization of the stacking gel, the gel cassette was mounted in the electrophoresis tank, the comb removed and electrode buffer added.

20 $\mu$ l of sample was loaded into wells in the stacking gel. The outermost lanes and any blank lanes were loaded with 20 $\mu$ l of double strength SDS-PAGE sample buffer. Gels were electrophoresed at 90V while the samples were travelling in the stacking gel and 150V while in the separating gel. Following electrophoresis, gels were removed for silver staining.

#### 5.2.2.5 Silver staining of polyacrylamide gels

Lipopolysaccharide was visualized on polyacrylamide using the method described by Hancock and Poxton (1988) as a modification of the previous silver staining methods of Tsai and Frasch (1982), Oakley *et al* (1980) and Hitchcock and Brown (1983).

#### 5.2.2.6 Serum sensitivity assay

##### 5.2.2.6a Serum Collection

Serum used as a source of complement was obtained from whole venous blood. The collected blood was allowed to clot at room temperature for 30 mins and then left on ice for 6 hours. The separated serum was centrifuged at 800 x g for 15 mins to remove suspended erythrocytes and stored until required in liquid nitrogen.

##### 5.2.2.5b Preparation of Bacterial Cells for serum sensitivity assay

Bacteria were grown in either nutrient broth (200 ml) or Osmolite (200 ml) at 25°C for 24 hours. Bacteria were harvested at 8000 x g for 15 min and washed once in sterile

0.01 M PBS and once in Complement Fixation Test Buffer (CFTB; Oxoid). Then harvested bacteria were resuspended in CFTB to give a concentration of approximately  $9.00 \log \text{cfu ml}^{-1}$ .

#### 5.2.2.6c Serum sensitivity assay protocol

Bacteria used in the serum sensitivity assay were prepared in the manner described in section 5.2.2.6b. CFTB (Control), CFTB+10% Serum or CFTB+10% heat-inactivated serum (2 ml in 2.5 ml sterile cryovials) were inoculated with approximately  $5.00 \log \text{cfu ml}^{-1}$  of the bacterial cultures suspended in CFTB (Section 5.2.2.6b) and incubated for 40 min at  $37^{\circ}\text{C}$  with rotation. Samples ( $100\mu\text{l}$ ) were removed at regular intervals and spread directly onto three agar plates or serially diluted 1:40 or 1:50 in CFTB and spread onto agar plates in triplicate. Plates were incubated at  $37^{\circ}\text{C}$  for 24 hours. Heat inactivation of serum was for 30 min at  $56^{\circ}\text{C}$ . Results were expressed as a mean of three independent experiments with its standard error of the mean in  $\log \text{cfu ml}^{-1}$ . Results were analysed using paired and unpaired students t-test.

#### 5.2.2.7 Cell culture

THP-1 cells were routinely cultured in "10% RPMI" which consisted RPMI 1640 media (Sigma) supplemented with 10% Foetal Calf Serum (FCS; Sigma) and 1mM L-glutamine (BDH). THP-1 cells were incubated at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  atmosphere and maintained twice weekly in fresh media (10% RPMI) supplemented with  $100\mu\text{g ml}^{-1}$  penicillin G (Sigma) and  $100\mu\text{g ml}^{-1}$  streptomycin sulphate (Sigma).

L929 murine fibroblasts were routinely cultured in "10% DMEM" which consisted of Dulbecco's Modified Essential Medium supplemented (DMEM) with 10% FCS and 1mM L-glutamine. L929 cells were also incubated at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  atmosphere and maintained twice weekly in fresh media (10% DMEM) supplemented with  $100\mu\text{g ml}^{-1}$  penicillin G and  $100\mu\text{g ml}^{-1}$  streptomycin sulphate.



#### 5.2.2.8 Enumeration of cells

Murine peritoneal macrophages and THP-1 cells were enumerated by removing a 50µl sample from suspended cells, diluting it in 450µl white cell diluting fluid (0.01% crystal violet in 1% acetic acid) and counting cells in a haemocytometer.

L929 were enumerated by diluting a 250µl sample of suspended cells 1:1 with a 0.1% solution of Trypan Blue (Sigma) in de-ionised water diluted 1:10 in 0.01M PBS and counting in a haemocytometer.

#### 5.2.2.9 Collection of murine peritoneal macrophages

Mice were killed by cervical dislocation. The peritoneal cavity was injected with 5 ml of pyrogen free 0.01M PBS and massaged to release the macrophages. These were recovered using a 0.8mm gauge needle and deposited into a plastic universal. The washings were centrifuged at 300g for 10 min to pellet the macrophages and the supernatant was discarded. The pellets were resuspended in a known volume of 10% DMEM without antibiotics. Cells were enumerated using white cell diluting fluid (Section 5.2.2.8) and then adjusted to the required level.

#### 5.2.2.10 Harvesting of THP-1 and L929 cell lines

THP-1 were harvested by centrifugation at 300g and resuspended in fresh 10% RPMI without antibiotics. They were enumerated using the method described in Section 5.2.2.8.

L929 murine fibroblast cells were dislodged from the flasks using 0.1% trypsin (Gibco) or 0.1% trypsin-EDTA (Gibco) to avoid clumping of cells. They were incubated for 5 min at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Finally, they were washed, resuspended in 10% DMEM without antibiotics and enumerated using the method in Section 5.2.2.8.

#### 5.2.2.11 Stimulation of murine peritoneal macrophages to release nitric oxide

Murine peritoneal cells were harvested and enumerated as described in Sections 5.2.2.9 and 5.2.2.8. Cells were adjusted to  $2.0 \times 10^6 \text{ ml}^{-1}$  by resuspending in 10% DMEM without antibiotics. Cells (100 $\mu\text{l}$ /well) were placed in a 96 well tissue plates incubated for 1 hour at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. After incubation, non-adherent cells (lymphocytes) were gently removed by washing the cells with 10% DMEM without antibiotics. Free LPS extracts prepared using methods described in Section 5.2.2.2b were suspended in 10% DMEM without antibiotics to concentrations of either 2, 20 or 200 $\mu\text{g ml}^{-1}$ . 100 $\mu\text{l}$  of these LPS solution was added to three replicate wells containing macrophages. 100 $\mu\text{l}$  of media was added to 3 replicate wells of macrophages as a negative control. In addition, prepared LPS from *Salmonella minnesota* (Urayet, 1996) and *P. aeruginosa* serotype 10 (Sigma) were included in experiments as positive controls. Cell culture plates were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 24 hours. Supernatants were removed for direct assessment of nitric oxide.

#### 5.2.2.12 Preparation of whole bacterial cells for stimulation of human monocyte cell lines (THP-1)

Whole bacterial cells were used to stimulate nitric oxide and TNF $\alpha$  production in the human monocyte cell line (THP-1). 200 ml of milk-based enteral nutrient solutions and nutrient broth were inoculated with an approximately 5.00 log cfu ml<sup>-1</sup> inoculum of bacteria from an overnight culture (Section 2.2.2) and incubated at 25°C for 24 hours. Cultures were centrifuged at 8000 x g. The supernatants were discarded and the pelleted bacteria were washed twice using pyrogen free 0.01M PBS. Cells were resuspended in 10 ml of pyrogen free 0.01M PBS. 1 ml samples of resuspended bacteria were removed and serially diluted in sterile PBS and counted using the spread plate method (Jay, 1992). Bacteria were killed by adding 90 $\mu\text{l}$  of formalin and incubating at 25°C for 24 hours. Bacteria were then washed and resuspended in 10% RPMI without antibiotics to a concentration of  $2.5 \times 10^9 \text{ cfu ml}^{-1}$ .

#### 5.2.2.13 Stimulation of human monocyte cell line (THP-1) to release nitric oxide and Tumour Necrosis Factor

THP-1 cells were harvested and enumerated as described in sections 5.2.2.10 and 5.2.2.8. 100µl/well of THP-1 cells and 100µl/well of test bacterial suspensions in 10% RPMI without antibiotics (Section 5.2.2.11) were introduced to 96 well tissue culture plates. 10% RPMI without antibiotics was added to 3 replicate wells (100µl/well) of monocytes as a negative control. Tissue culture plates were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 24 hours. Plates were centrifuged at 2400 x g for 30 mins and the supernatants were removed for direct assessment of nitric oxide and TNFα.

#### 5.2.2.14 Nitric oxide assay

Nitric oxide was measured using a modification of the method of Stuehr and Marletta (1987) which measures nitrite, a stable product of nitric oxide. In brief, 100µl/ well of Greiss reagent (1:1 v/v mixture of 0.3% N-[1 naphthyl]ethylenediamine dihydrochloride [Sigma] in deionised H<sub>2</sub>O to 1% sulphanilamide [Sigma] in 5% phosphoric acid) was added to clean 96 well assay plates. To this an equal volume of each test supernatant (Sections 5.2.2.11 and 5.2.2.13) was added and incubated for 10 minutes at room temperature. Plates were read at 590 nm using a MR7000 micro-plate reader (Dynatech Laboratories). Standard samples (100µl/well) of sodium nitrite and a negative control of 10% DMEM or 10% RPMI were added to triplicate wells in each test plate. The standard samples of sodium nitrite were used to construct a standard curve and prepared by serially diluting (1:2) a 10mM sodium nitrite (Sigma) solution in 10% RPMI or 10% DMEM without antibiotics to give concentrations in the range 256 µmol ml<sup>-1</sup> to 1µmol ml<sup>-1</sup>. The results of each standard curve were calculated from the mean of three wells (with standard error) and linear regression was used to generate a line of best fit from which test results were calculated. Experiments using murine peritoneal macrophages and extracted LPS as a stimulus were repeated three times. The test results were expressed as a mean of the three independent experiments (with standard error of the mean) in µmol per 10<sup>5</sup> bacterial cells per 24 hours. Experiments using the THP-1 cell line

and whole bacterial cells as a stimulus were repeated twice. Test results from each of these experiments were presented separately and expressed as a mean of three wells, with its standard error, in  $\mu\text{mol}$  per  $10^5$  bacterial cells per 24 hours. Students t-tests were used to analyse data in all experiments.

#### 5.2.2.15 TNF $\alpha$ bioassay

L929 cells were cultured (Section 5.2.2.7), harvested (Section 5.2.2.10), enumerated (Section 5.2.2.8) and resuspended in antibiotic free growth media to  $3.5 \times 10^5$  cells  $\text{ml}^{-1}$ . 100 $\mu\text{l}$ /well of the suspended cells were placed in a 96 well tissue culture plate and incubated at 37°C in an incubator with a humidified atmosphere containing 5%  $\text{CO}_2$  for 24 hours. The growth media was discarded and replaced with 100 $\mu\text{l}$ /well of test supernatant (Section 5.2.2.13) diluted 1:1 with assay medium (DMEM containing 5% FCS, 1mM L-glutamine and 2 $\mu\text{g ml}^{-1}$  actinomycin D, Sigma). Plates were then incubated at 37°C in a humidified, 5%  $\text{CO}_2$  atmosphere for 24 hours. Following incubation, the supernatants from each well were removed and replaced with 50 $\mu\text{l}$ /well of crystal violet stain (0.5% crystal violet (Sigma) in 20% methanol in de-ionised water and filtered through Whatman No. 1 filter paper) for 2 minutes to stain surviving cells. Plates were washed very vigorously using tap water, dried thoroughly and then 50 $\mu\text{l}$ /well of 20% acetic acid was used to solubilize stained cells. Standard TNF $\alpha$  samples and negative controls were included on each plate. The standard TNF $\alpha$  samples were prepared by serially diluting 1:3 standard recombinant human TNF $\alpha$  (NIBSC, Hertfordshire, UK) in assay media (DMEM containing 5% FCS, 1mM L-glutamine and 1 $\mu\text{g ml}^{-1}$  actinomycin D) to give concentrations between 1000 IU  $\text{ml}^{-1}$  to 0.1 IU  $\text{ml}^{-1}$ . These were added to plates (100 $\mu\text{l}$ /well) and used to construct a standard curve. Negative controls containing 100 $\mu\text{l}$ /well of assay media per wells. 3 blanks containing no L929 cells were included on each plate and these were blanked on when spectrophotometric measurements were made. Plates were read at 570nm using a Dynatech MR 700 micro-plate reader (Dynatech Laboratories). The results of each standard curve were calculated from the mean of three wells (with standard error) and linear regression was used to generate a

line of best fit from which test results were calculated. Experiments were repeated twice and an example of results from each experiment were calculated separately, as the mean of three wells (with standard error) in IU ml<sup>-1</sup>. Students t-tests were used to analyse the data.

5.2.2.17 The effect of adding sub-lethal concentrations of oil of fennel, parabens and combinations of these antimicrobial chemicals on the expression of O-polysaccharide from *P. aeruginosa* grown in nutrient broth and Osmolite

2 x 500 ml of either nutrient broth or Osmolite were supplemented with either 0.5% oil of fennel, 0.1% methyl parabens or a combination of these preservatives and inoculated with 10 ml of an overnight culture (Section 2.2.2). Cultures were incubated for 24 hours at 25°C. Cells were then harvested and the LPS extracted and prepared for SDS-PAGE using the methods in Section 5.2.2.1 and 5.2.2.2. Gels were prepared and stained using the methods in Section 5.2.2.3 and 5.2.2.4.

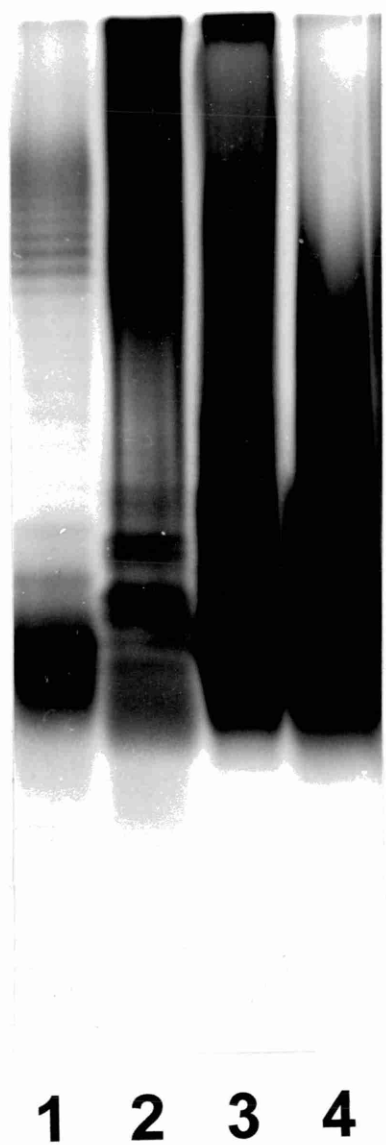
## 5.3 RESULTS

### 5.3.1 Comparison of O-polysaccharide side chain heterogeneity in bacteria grown in laboratory media to those grown in milk-based enteral nutrient solutions

Polyacrylamide gel electrophoresis can be used to visualize the heterogeneity of O-polysaccharide side chains of lipopolysaccharide. This study used the technique to compare the heterogeneity of O-polysaccharide from bacteria of a single strain grown in either in laboratory media or milk-based enteral nutrient solutions. Smooth LPS when analysed on a polyacrylamide gel appears as a series of bands. Each band represents lipopolysaccharide molecules with increasing numbers of repeating oligosaccharide units in their O-polysaccharide side chains. Thus, bands at the bottom of the gel are lipopolysaccharide molecules with short chain length O-polysaccharide while bands at the top of the gel are lipopolysaccharide with longer chains of O-polysaccharide.

#### 5.3.1.1 Selection of methods and bacterial species for preparing extracts of lipopolysaccharide

In initial experiments, lipopolysaccharide was prepared using the Proteinase K digestion method. This technique disrupts whole bacterial cells by boiling them in SDS-PAGE sample buffer for 10 minutes. Incubation with the Proteinase K should remove all proteinaceous impurities from the sample leaving the lipopolysaccharide which can be visualized using polyacrylamide electrophoresis and silver staining. However, this technique did not produce clear banding patterns after several repetitions (results not shown) and so was not used in further experiments. Subsequently, the hot-aqueous phenol method was selected to purify lipopolysaccharide from whole bacterial cells. This was more successful. Extracts of lipopolysaccharide were prepared from *E. coli*, *Ent. cloacae*, *K. aerogenes* and *P. aeruginosa* grown in nutrient broth and analysed on a 14% acrylamide gel (Figure 5.1). The result showed banding patterns for lipopolysaccharide extracted from *E. coli* and *P. aeruginosa* but clear bands were not



**Figure 5.1:** SDS-PAGE gels of hot aqueous-phenol extracts of LPS from bacteria grown in nutrient broth. Lane 1: *E. coli*; Lane 2: *P. aeruginosa*; Lane 3: *K. aerogenes*; Lane 4: *Ent. cloacae*.

seen in lipopolysaccharide extracted from *Ent. cloacae* and *K. aerogenes*. Therefore, *E. coli* and *P. aeruginosa* were selected for further experiments using different growth media.

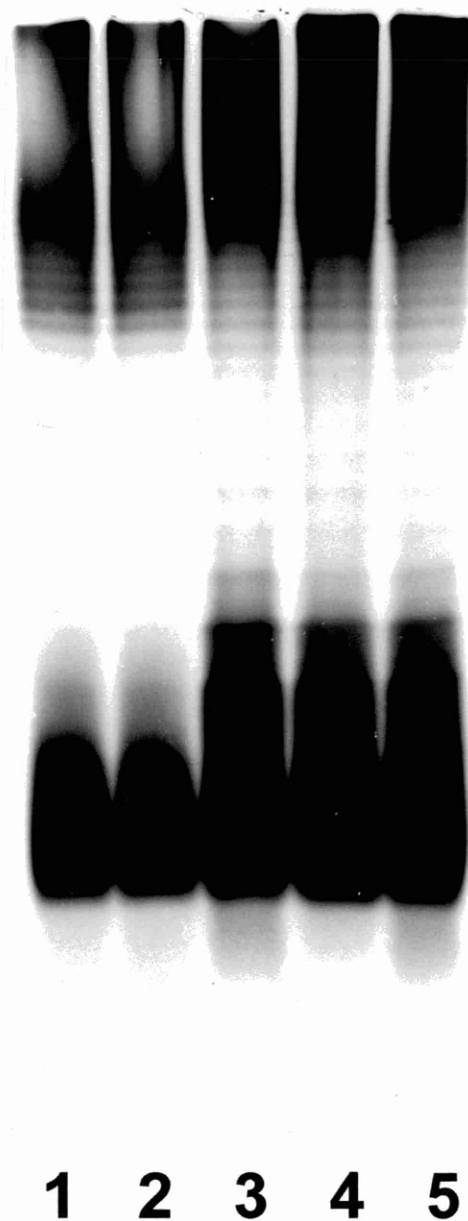
#### 5.3.1.2 Comparison of O-polysaccharide side chain heterogeneity of *E. coli* grown in laboratory media compared to milk-based enteral nutrient solutions.

The results indicated that *E. coli* grown in nutrient broth produced a greater proportion of its lipopolysaccharide with long chain O-polysaccharide, while *E. coli* grown in the milk-based enteral nutrient solutions (Osmolite, Fortisip and Pulmocare) produced a greater proportion of lipopolysaccharide with short chain O-polysaccharide (Figure 5.2). In addition, there was greater heterogeneity in the O-polysaccharide chain lengths of lipopolysaccharide from bacteria grown in the milk-based enteral nutrient solutions. This was evident because of the increased number and amounts of bands of intermediate molecular weight in lanes 3, 4 and 5. There was no differences between in O-polysaccharide heterogeneity of Osmolite, Fortisip or Pulmocare.

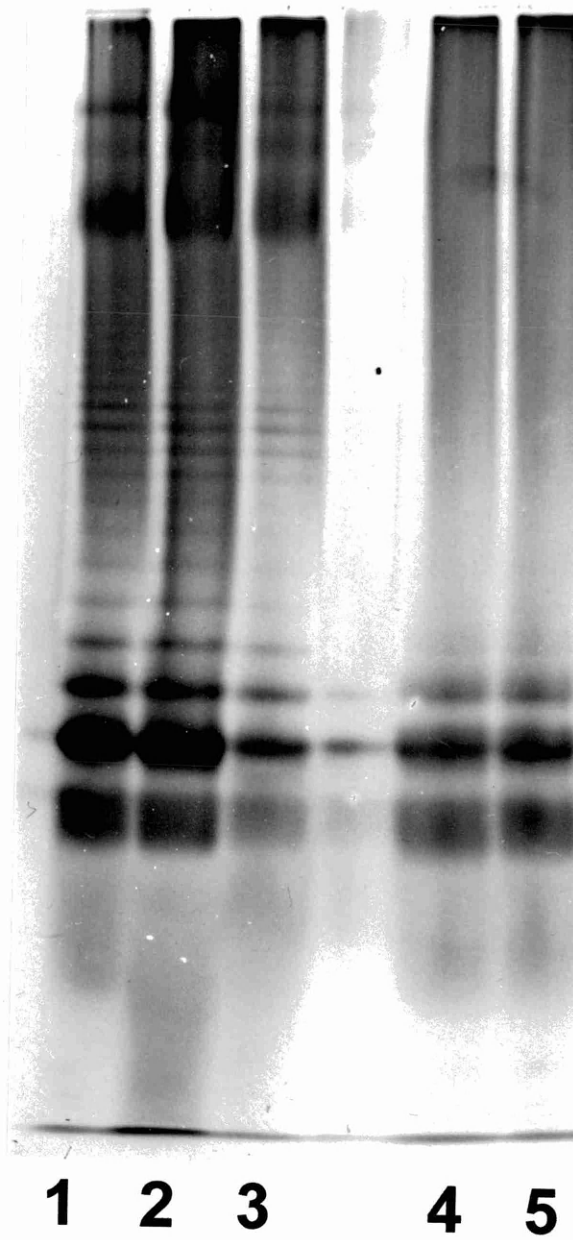
#### 5.3.1.3 Comparison of O-polysaccharide side chain heterogeneity of *P. aeruginosa* grown in laboratory media compared to milk-based enteral nutrient solutions

The results show that *P. aeruginosa* grown in nutrient broth produce little or no lipopolysaccharide with O-polysaccharide side chains of intermediate molecular weight (Figure 5.3). In contrast, there was lipopolysaccharide with O-polysaccharide side chains of intermediate molecular weight when *P. aeruginosa* was cultured in any of the milk-based enteral nutrients solutions. The profiles of lipopolysaccharide from *P. aeruginosa* grown in the milk-based enteral nutrient solutions were all similar.





**Figure 5.2:** SDS-PAGE gel of hot aqueous-phenol extracts of *E. coli* grown in laboratory media and milk-based enteral nutrient solutions. Lane 1 and 2: *E. coli* grown in nutrient broth; Lane 3: *E. coli* grown in Osmolite; Lane 4: *E. coli* grown in Fortisip; Lane 5: *E. coli* grown in Pulmocare.



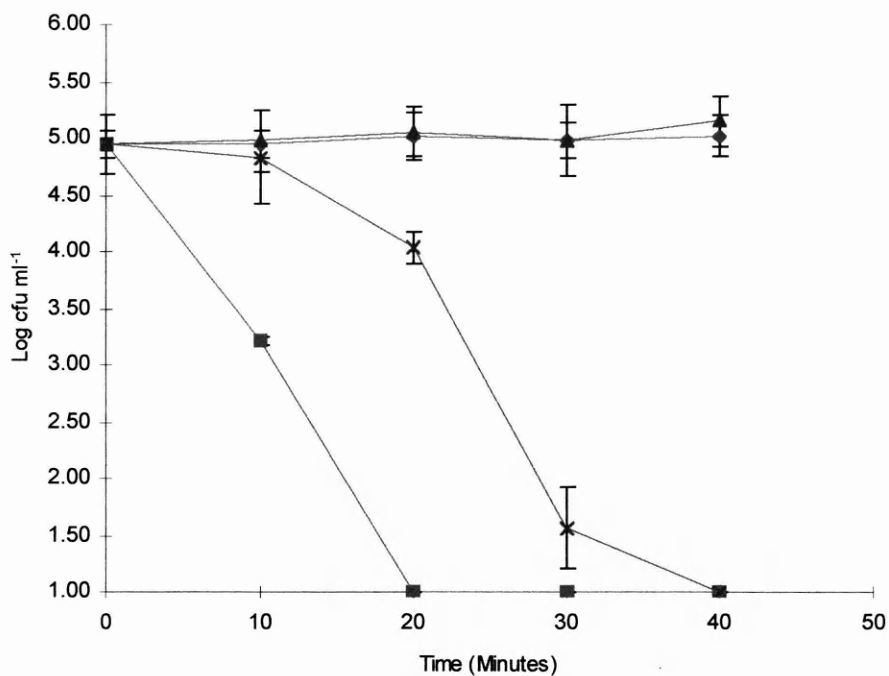
**Figure 5.3:** SDS-PAGE gels of hot aqueous-phenol extracts of *P. aeruginosa* grown in laboratory media and milk-based enteral nutrient solutions. Lane 1: *P. aeruginosa* grown in Osmolite; Lane 2: *P. aeruginosa* grown in Fortisip; Lane 3: *P. aeruginosa* grown in Pulmocare; Lane 4 and 5: *P. aeruginosa* grown in nutrient broth.

### 5.3.2 Serum resistance of bacteria grown in nutrient broth compared to milk-based enteral nutrient solutions

The serum resistance of *E. coli* grown in nutrient broth was compared to the serum sensitivity of *E. coli* grown in Osmolite, a milk-based enteral nutrient solution (Figure 5.4). In each experiment, survival in 10% serum was compared to a control which contained no serum. *E. coli* grown in each type of growth environment were also incubated with 10% heat inactivated serum to see if serum killing was complement mediated.

There were no significant changes in the numbers of *E. coli* in either of the controls during the experiment and there were no significant differences between the initial numbers of *E. coli* in any of the tubes at the beginning of the experiments. In addition, there was no significant change in *E. coli* numbers when incubated with 10% heat inactivated serum, indicating that killing was complement mediated (Data not shown).

In the presence of 10% serum, a significant reduction ( $p < 0.001$ ) in the numbers of *E. coli* grown in nutrient broth (compared to the control) was observed after just 10 minutes. Numbers decreased from an initial concentration of  $4.95 \log \text{cfu ml}^{-1}$  to  $3.21 \log \text{cfu ml}^{-1}$ . After 20 minutes numbers of *E. coli* cultured in this medium were undetectable ( $< 1.00 \log \text{cfu ml}^{-1}$ ). This was in contrast to *E. coli* grown in Osmolite which were more resistant to the killing effects of serum. There was no significant reduction in the numbers of *E. coli* cultured in Osmolite until 20 minutes ( $p < 0.01$ ), at which point numbers had decreased from  $4.95 \log \text{cfu ml}^{-1}$  to  $4.04 \log \text{cfu ml}^{-1}$ , a decrease of 88%. Between 20 and 30 minutes numbers of *E. coli* cultured in Osmolite decreased a further 12% and it was not until the 40 minute time interval that numbers became undetectable. Therefore in the presence of 10% serum, numbers of *E. coli* grown in nutrient broth were significantly lower than numbers of *E. coli* grown in Osmolite at 10 minutes ( $p < 0.05$ ), 20 minutes ( $p < 0.001$ ) and 30 minutes ( $p < 0.05$ ).



**Figure 5.4:** The serum sensitivity of *E. coli* grown either in laboratory media or milk-based enteral nutrient solution. Green diamond: *E. coli* grown in nutrient broth, no serum; Green square: *E. coli* grown in nutrient broth, 10% serum; Blue diamond: *E. coli* grown in Osmolite, no serum; Blue cross: *E. coli* grown in Osmolite, 10% serum.

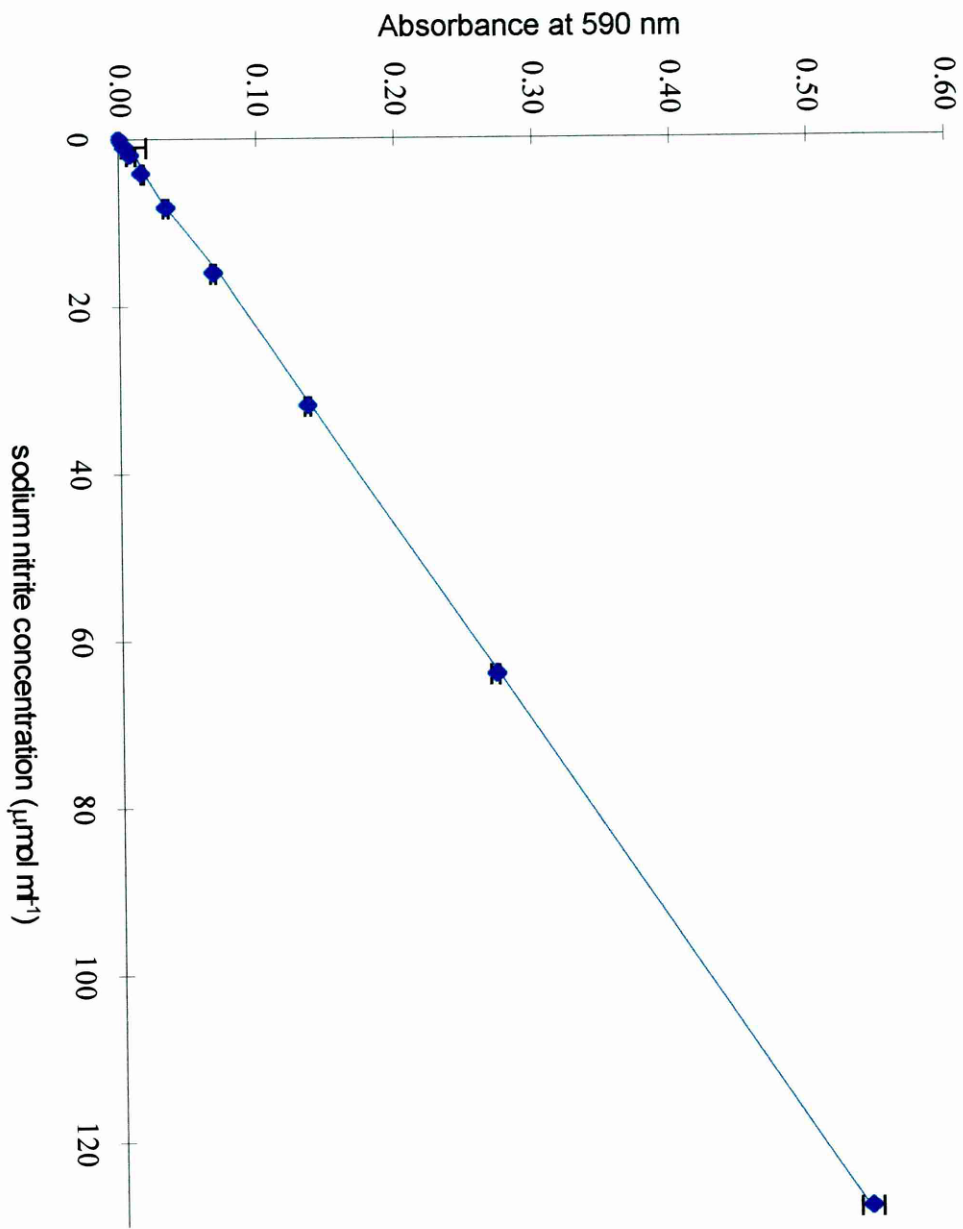
### 5.3.3 Production of inflammatory mediators by macrophages and monocytes in response to lipopolysaccharide and whole bacterial cells

#### 5.3.3.1 Production of nitric oxide by murine peritoneal macrophages in response to free lipopolysaccharide extracts

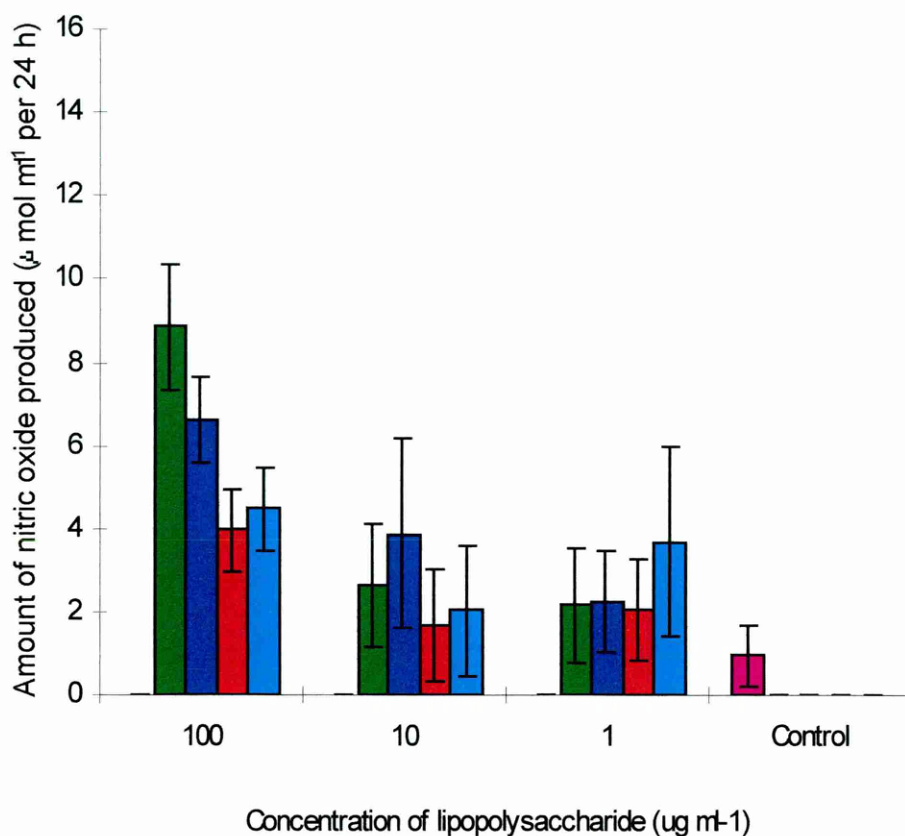
Nitric oxide production by murine peritoneal macrophages in response to hot-phenol extracts of lipopolysaccharide from *P. aeruginosa* grown in nutrient broth, *P. aeruginosa* grown in Osmolite, *P. aeruginosa* Serotype 10 (Sigma Chemicals Ltd.) and *S. minnesota* used in the work of Urayet (1996) was measured. Nitric oxide production by murine peritoneal macrophages in the absence of lipopolysaccharide was also measured to establish a baseline. Experiments were conducted over a 24 hour period and the amount of nitric oxide produced by  $10^6$  macrophages was calculated by relating the amount of nitrite present in the supernatant to a sodium nitrite standard curve (Figure 5.5). Experiments were repeated three times and the mean of these results are presented in Figure 5.6.

Murine macrophages produced  $0.95 \mu\text{mol}$  nitric oxide per  $10^6$  cells per 24 hours. Lipopolysaccharide from all the different sources induced significantly ( $p < 0.05$ ) more nitric oxide than this baseline when used at a concentration of  $100 \mu\text{g ml}^{-1}$ . However, when the LPS were used at the lower concentrations ( $10$  and  $1 \mu\text{g ml}^{-1}$ ) nitric oxide production was not significantly different from the baseline.

Amounts of nitric oxide produced by the murine macrophages in response to stimulus were low ( $< 8.86 \mu\text{mol}$  per  $10^6$  cells per 24 hours). There was no significant differences, at any of the LPS concentrations, between the amounts of nitric oxide produced by macrophages in response to the LPS of *P. aeruginosa* grown in nutrient broth or the LPS of *P. aeruginosa* grown in Osmolite. Neither were the amounts of nitric oxide produced in response to any of the concentrations of the LPS of *S. minnesota* or the LPS of *P. aeruginosa* serotype 10, significantly different to the amount of nitric oxide



**Figure 5.5:** An example of sodium nitrate standard curve used in nitric oxide assays. Each point represents the mean of three independent experiments with its standard error of the mean.



**Figure 5.6:** Nitric oxide production by murine peritoneal macrophages in response to hot aqueous-phenol extracts of lipopolysaccharide. Green: *P. aeruginosa* grown in nutrient broth; Blue: *P. aeruginosa* grown in Osmolite; Red: *S. minnesota* (Positive control); Green: Standard *P. aeruginosa* serotype 10 (Positive control); Magenta: Negative control, no LPS. Each point represents of the mean of three independent experiments with its standard error of the mean.

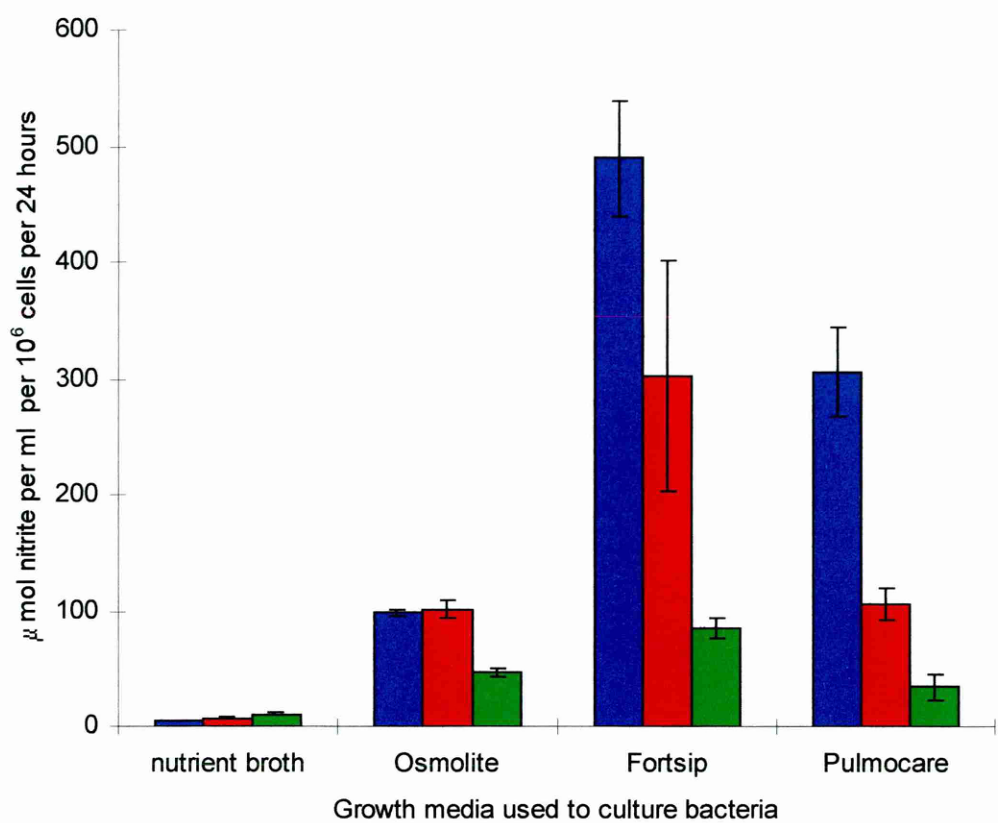
produced in response to LPS from *P. aeruginosa* culture in either nutrient broth or Osmolite.

#### 5.3.3.2 Production of nitric oxide by a human monocyte cell line in response to whole bacterial cells

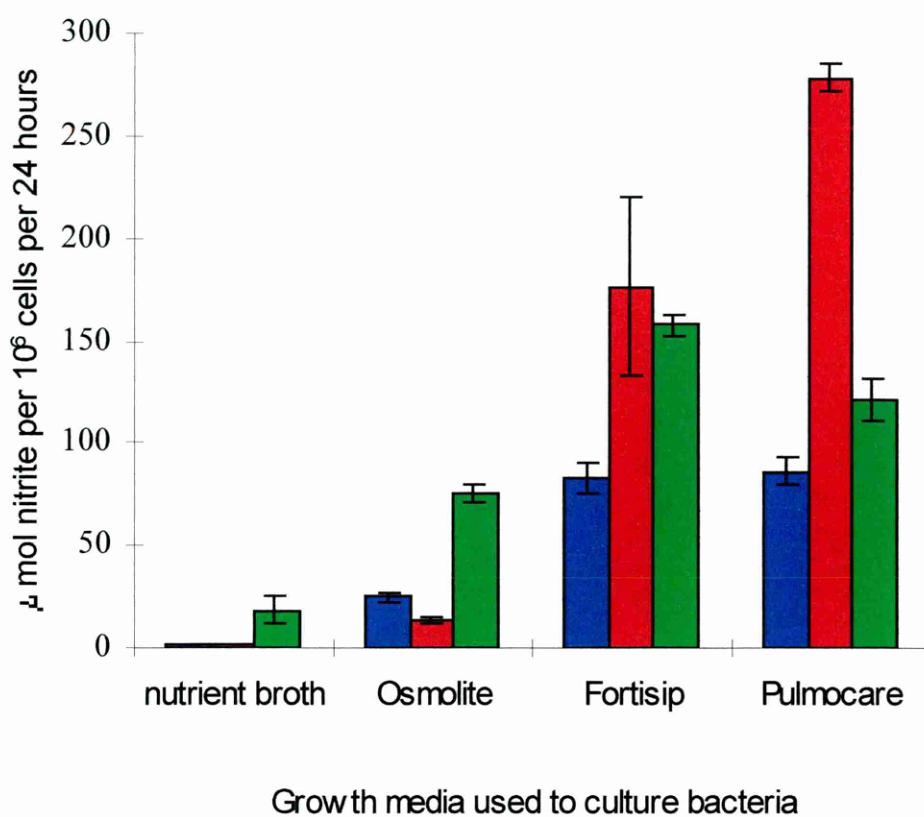
The potential of whole *E. coli*, *Ent. cloacae* and *P. aeruginosa* cells grown either in nutrient broth or milk-based enteral nutrient solution (Osmolite, Fortisip, Pulmocare) to stimulate nitric oxide production by human monocytes was investigated using the THP-1 human monocyte cell line. Experiments were conducted over a 24 hour period and the amount of nitric oxide produced by  $10^6$  monocytes in response to  $10^8$  bacteria was calculated by relating the amount of nitrite present in the supernatant (a stable end-product of nitric oxide synthesis) to a sodium nitrite standard curve (Figure 5.5). Nitric oxide production by the THP-1 cell line in the absence of whole cells was also measured to establish a baseline. Experiments were repeated twice and both sets of results are shown (Figures 5.7 and 5.8).

In experiment 1, all bacterial strains grown in either nutrient broth or milk-based enteral solutions stimulated the THP-1 cells to produce significantly more ( $p < 0.05$ ) nitric oxide than the baseline level ( $1.98 \mu\text{mol per } 10^6 \text{ per } 24 \text{ hours}$ ). In contrast, only the bacteria grown in milk-based enteral nutrient solutions stimulated cells to produce significantly more nitric oxide than the baseline level of  $1.30 \mu\text{mol per } 10^6 \text{ per } 24 \text{ hours}$ . Both experiments indicated that there was significantly more nitric oxide ( $p < 0.001$ - $p < 0.05$ ) produced by THP-1 cells in response to bacteria grown in milk-based enteral nutrient solutions, compared to bacteria cultured in nutrient broth. In addition, both experiments indicated that growth in different milk-based enteral nutrient solutions also affected the potential of bacteria to stimulate THP-1 cells to produce nitric oxide. Both experiments also showed that bacteria grown in Osmolite stimulated THP-1 cells to produce less nitric oxide than bacteria grown in other milk-based enteral nutrient





**Figure 5.7:** Experiment one, Nitric oxide production by a human monocyte cell line in response to whole bacteria grown either in laboratory media or milk-based enteral nutrient solution. Blue: *E. coli*; Red: *Ent. cloacae*; Green: *P. aeruginosa*. Each bar is the mean of three wells from one experiment with its standard error of the mean.



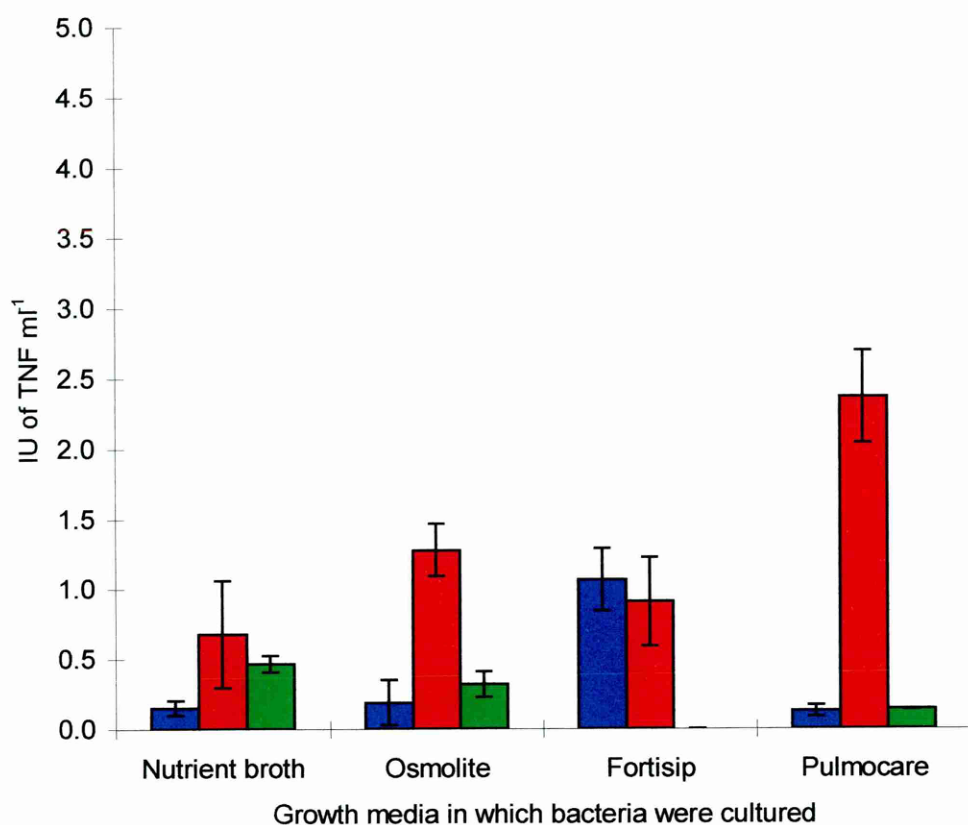
**Figure 5.8:** Experiment two, nitric oxide production by a human monocyte cell line in response to whole bacteria grown either in laboratory media or milk-based enteral nutrient solutions. Blue: *E. coli*; Red: *Ent. cloacae*; Green: *P. aeruginosa*. Each bar is the mean of three wells from one experiment with its standard error of the mean.

solutions. However, there was no consistent pattern in experiments to the amounts of nitric oxide produced in response to bacteria grown in either Fortisip or Pulmocare. Neither was there consistency in the patterns of nitric oxide produced in response to different strains of bacteria.

#### 5.3.3.3 Production of Tumour Necrosis Factor by a human monocyte cell line in response to whole bacterial cells grown in laboratory media and enteral nutrient solutions

The potential of whole *E. coli*, *Ent. cloacae* and *P. aeruginosa* cells grown in nutrient broth or milk-based enteral nutrient solutions (Osmolite, Fortisip or Pulmocare) to stimulate nitric oxide production by human monocytes was investigated using the THP-1 monocyte cell line. Supernatants from  $10^6$  monocyte cells incubated with  $10^8$  bacterial cells were harvested at 24 hours and the amount of TNF $\alpha$  calculated by relating the amount of TNF $\alpha$  present in the culture supernatant to a standard curve of TNF $\alpha$  standards. Experiments were repeated twice and a representative set of results is shown (Figure 5.9).

In general, small amounts ( $<2.36$  IU ml $^{-1}$ ) of TNF $\alpha$  were measured in the supernatants stimulated with bacteria grown in nutrient broth and milk-based enteral nutrient solutions. In most cases, there was no significant difference between the amounts of TNF $\alpha$  measured in supernatants from cells stimulated with bacteria grown in nutrient broth or in supernatants from cells stimulated with bacteria grown in milk-based enteral nutrient solutions.



**Figure 5.9:** Production of TNF $\alpha$  by a human monocyte cell line in response to whole bacterial cells grown in either laboratory media or milk-based enteral nutrient solution. Blue: *E. coli*; Red: *Ent. cloacae*; Green: *P. aeruginosa*. Each bar represents the mean of three wells from one experiment with its standard error of the mean.

#### **5.3.4 Effect of adding sub-lethal concentrations of oil of fennel, parabens and combinations of these antimicrobial agents on the expression of O-polysaccharide from *P. aeruginosa*.**

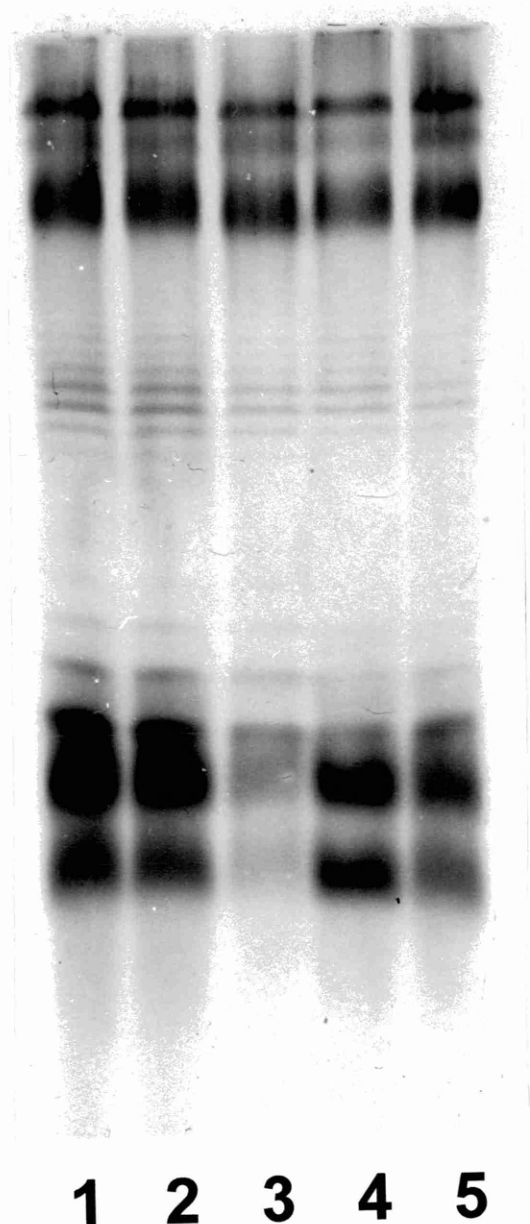
*P. aeruginosa* cells were grown in nutrient broth or Osmolite supplemented with 0.5% oil of fennel, 0.1% methyl parabens or a combination of 0.5% oil of fennel and 0.1% methyl parabens.

##### **5.3.4.1 Effect of adding sub-lethal concentrations of oil of fennel, parabens and combinations of these antimicrobial agents on the expression of O-polysaccharide from *P. aeruginosa* growing in nutrient broth**

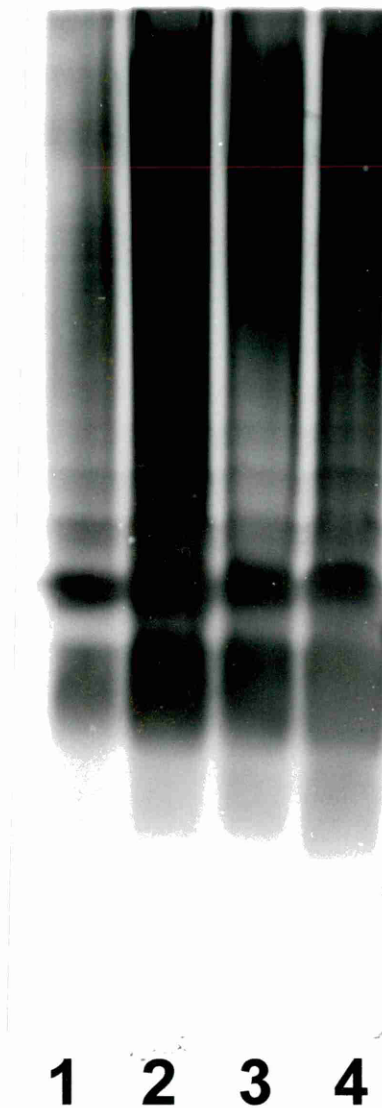
The results show that lipopolysaccharide with long and short chain O-polysaccharide was produced by *P. aeruginosa* in nutrient broth and broth supplemented with sub-lethal concentrations of antimicrobial agents (Figure 5.10). The presence of 0.5% oil of fennel, 0.1% methyl parabens or combinations of these concentrations of antimicrobial agents did not alter the heterogeneity of lipopolysaccharide expression. In addition, there were no obvious changes in the proportions of lipopolysaccharide molecules with O-polysaccharide of different chain length.

##### **5.3.4.2 Effect of adding sub-lethal concentrations of oil of fennel, parabens and combinations of these antimicrobial agents on the expression of O-polysaccharide from *P. aeruginosa* growing in Osmolite**

Results show that lipopolysaccharide molecules with long, medium and short chain O-polysaccharide were produced by *P. aeruginosa* grown in Osmolite, or in Osmolite supplemented with sub-lethal concentrations of preservatives (Figure 5.11). The presence of 0.5% oil of fennel, 0.1% methyl parabens or a combination of these antimicrobial agents in Osmolite did not alter the heterogeneity of lipopolysaccharide expression nor alter the proportions of lipopolysaccharide with different O-polysaccharide chain lengths.



**Figure 5.10:** SDS-PAGE gel of lipopolysaccharide from *P. aeruginosa* grown in nutrient broth supplemented with preservatives. Lane 1: No preservatives; Lane 2: 0.5% oil of fennel; Lane 3: 0.1% methyl parabens; Lane 4: 0.5% oil of fennel and 0.1% methyl parabens.



**Figure 5.11:** SDS-PAGE gel showing lipopolysaccharide from *P. aeruginosa* grown in Osmolite supplemented with preservatives. Lane 1 and 2: No preservatives; Lane 3: 0.5% oil of fennel; Lane 4: 0.1% methyl parabens; Lane 5: 0.5% oil of fennel and 0.1% methyl parabens.

## 5.4 DISCUSSION

The first aim of this Chapter was to investigate whether the phenotypic expression of lipopolysaccharide, as determined by SDS-PAGE, in enteral nutrient solutions was similar to that already established by conventional means in laboratory media. The quickest method of preparing both rough and smooth LPS for SDS-PAGE is using Proteinase K to enzymically remove proteinaceous contaminants (Hancock and Poxton, 1988). This technique was also selected in the first place because it requires a relatively small amount of bacterial cell mass to produce enough LPS for SDS-PAGE. Other available techniques for LPS extractions, such as the hot-phenol extraction of Westphal and Luderitz (1954) as described by Hancock and Poxton (1988) or extraction by the phenol-chloroform petroleum spirit mixture developed by Galanos *et al* (1969) cited by Hancock and Poxton (1988), required much larger amounts of bacterial cell mass. Therefore, Proteinase K was the most suitable method for extracting LPS from bacteria grown in fruit-based enteral nutrient solution (Fortijuce) where experiments from Chapter 3 showed growth was poor and thus, bacterial concentrations at 24 hours were low. However, this technique could not be used because of the high proportion of insoluble proteins and unidentified matter which were carried over in the harvested bacterial mass from milk-based enteral nutrient solutions. This material could not be removed by repeated washes in PBS or by increasing the concentration of Proteinase K and lengthening the period of incubation from 1 to 24 hours. SDS-PAGE gels using LPS from these extractions were too poor to distinguish any banding patterns and so the Hot Phenol Technique was selected to investigate O-polysaccharide heterogeneity. Unfortunately, this meant that the amount of Fortijuce required to culture enough bacterial mass for LPS extraction made further experimentation with this medium prohibitive.

The results from this study have shown that *E. coli* and *P. aeruginosa* both produced populations of lipopolysaccharide with heterogeneous O-polysaccharide chain length. These are findings similar to Goldman and Leive (1984) who found *E. coli* O111:B4



produced a heterogeneous O-polysaccharide with up to 40 oligosaccharide units or Rivera and McGroarty (1989) and McGroarty and Rivera (1990) who found *P. aeruginosa* produced two chemotypes of LPS with considerable heterogeneity observed in the O-polysaccharide chain length of both A and B band LPS. More importantly, the results from this study showed that the proportions of LPS with different O-polysaccharide chain lengths varied according to whether bacteria were grown in the milk-based enteral nutrient solutions or laboratory media.

Such phenotypic changes in the expression of O-polysaccharide side chains of lipopolysaccharide can be as a result of differences in growth temperature (Schlecht and Mayer, 1994), growth rate (Dodds *et al*, 1987) and nutrient composition of the media (Weiss *et al*, 1986; Kelly *et al*, 1989; McGroarty and Rivera, 1990; Allan and Poxton, 1994). Bacteria were all grown at the same temperature. However, although the results of Chapter 3 show that the growth rate of *E. coli* in nutrient broth and milk-based enteral nutrient solutions at 24 hours were similar, the growth rates of *P. aeruginosa* in nutrient broth and milk-based enteral nutrient solutions were different at 24 hours. Bacterial cells in the early log phase, which are multiplying quickly, tend to produce a greater proportion of LPS with shorter O-polysaccharide chains (Dodds *et al*, 1987). Results from Chapter 3 show that the growth rate of *P. aeruginosa* in nutrient broth was slower than in milk-based enteral feeds which would indicate a longer average O-antigen chain length in those cells growing in nutrient broth. However, the results from the SDS-PAGE indicate the opposite, with *P. aeruginosa* grown in nutrient broth having a tendency to produce a shorter average O-antigen chain length. Therefore, the effect of growth rate is unlikely to be important in explaining the differences in O-polysaccharide chain lengths of *P. aeruginosa* in these experiments.

Nutrient composition of the media seems to be indicated as the most important factor responsible for the different phenotypes of LPS produced by *P. aeruginosa* and *E. coli* in milk-based enteral nutrient solutions compared to nutrient broth. Growth media rich in

nutrients, particularly carbon sources such as glucose, are known to increase the fraction of *E. coli* LPS containing O-polysaccharide and also cause an increase in the lengths of attached O-antigen (Weiss *et al*, 1986). On the other hand, in media where carbon and magnesium is restricted short chains are the prevalent type of O-polysaccharide in *E. coli* LPS (Dodds *et al*, 1987; Nelson *et al*, 1991). Enteral nutrient solutions are rich in carbon sources, nitrogen, phosphates and essential metal ions such as magnesium and iron. Nutrient broth is a standard laboratory media for a wide range of bacterial species but is not very rich in nutrients. Therefore, it would be expected that *E. coli* grown in the milk-based enteral formulas, as opposed to nutrient broth, would produce a greater proportion of LPS with a long chain O-polysaccharide. The results from this study showed that this was not the case and in fact the opposite was true. This may be because not all the nutrients were in a form able to be utilized by the *E. coli* strain used in the experiment. In future studies, the distribution and size heterogeneity of *E. coli* O-polysaccharide chains may be investigated further by using flow cytometry.

*P. aeruginosa* also changed the phenotypic expression of its LPS in response to the nutrient composition of its growth environment. McGroarty and Rivera (1990) showed that the production of longer chain O-polysaccharide on B-band LPS is reduced in the presence of low pH and low phosphate concentrations or high concentrations of NaCl, MgCl<sub>2</sub>, glycerol and sucrose, while the size of O-polysaccharide from A-band LPS (Common antigen) is unaffected in these restricted conditions. In addition, the proportion of A-band to B-band LPS may also increase in these circumstances (Rivera and McGroarty, 1990; Makin and Beveridge, 1996). In contrast to *E. coli*, *P. aeruginosa* grown in milk-based enteral nutrient solutions produced LPS with O-polysaccharide of intermediate chain length which was not present in *P. aeruginosa* grown in nutrient broth. This may be explained by the rich composition of enteral nutrient solutions stimulating the production of longer chain LPS or by there being a greater proportion of A-band LPS, which has shorter chain O-polysaccharide than B-band LPS (Lam *et al*, 1992), being produced in the *P. aeruginosa* grown in nutrient broth. However, this latter

hypothesis would need to be confirmed by Western Blotting because SDS-PAGE does not discriminate between A- and B-band LPS. Unfortunately, monoclonal antibodies against this strain of *P. aeruginosa* were not available to this study.

It is clear that the phenotype of LPS produced by *E. coli* and *P. aeruginosa* is different when these microorganisms are grown in milk-based enteral nutrient solutions compared to standard methods in laboratory media. Therefore from a purely structural point of view, experiments in laboratory media are not representative of bacteria growing in enteral nutrient solutions.

The second aim of this Chapter was to investigate whether aspects of Gram-negative bacterial pathogenicity mediated by lipopolysaccharide were altered when bacteria were grown in enteral nutrient solutions compared with conventional means in laboratory media. In order to achieve this, the serum resistance of *E. coli* grown in nutrient broth was compared to that of *E. coli* grown in Osmolite. There are no agreed definitions for serum sensitivity and no standard test method which makes comparisons between studies difficult (Crokaert *et al*, 1992). Therefore, parameters used in this study (10% serum, 37 °C) and the method for harvesting serum were previously used in a similar study by Allan and Poxton, (1994)

The results showed that no significant inactivation of bacteria grown in either media occurred when using heat-treated serum, indicating that complement was the most important serum component involved in the inactivation of *E. coli*. Complement inactivation of Gram-negative bacteria is dependent on the formation of and insertion of the membrane attack complex (C5b-9) into the outer membrane (Joiner *et al*, 1984; Grossman *et al*, 1987; Frank *et al*, 1987). O-polysaccharide (Porat *et al*, 1992), outer membrane proteins (Weisser and Gotschlich, 1991) and capsular polysaccharide (Taylor *et al*, 1981) are known to be important cell surface structures of *E. coli* which mediate resistance to complement inactivation. The O-polysaccharide portion of LPS molecules

is generally considered to be the most important structure involved. Long chain O-polysaccharide preferentially binds C3 resulting in the formation of the membrane attack complex far away from the outer membrane of the bacteria (Joiner *et al*, 1986; Grossman *et al*, 1987). In addition, the distribution of O-polysaccharide chains has been found to be important in conferring resistance to serum. Grossman and co-workers (1987) found that the survival of *S. montevideo* was associated with an average O-polysaccharide chain length of >5 units and with cells that had 20-23% of their LPS with O-polysaccharide of >14 units in length. They also found that the distribution of LPS with O-polysaccharide of chain length >14 units influenced the serum resistance of *S. montevideo* by forming a steric barrier to C3 deposition on LPS molecules with short chain O-polysaccharide.

The results from this study show that *E. coli* grown in milk-based enteral nutrient solution (Osmolite) were more resistant to serum than those *E. coli* grown in nutrient broth. However, SDS-PAGE gels of O-polysaccharide from *E. coli* grown in each media showed that cells grown in Osmolite produced a greater proportion of their LPS with short-chain O-polysaccharide compared to those grown in nutrient broth. On this basis, it would be anticipated that *E. coli* grown in Osmolite would have less resistance to serum killing than *E. coli* grown in nutrient broth. Therefore factors other than differences in the phenotypic expression of O-polysaccharide may be responsible for these observations.

Outer membrane proteins and capsules are also structures whose phenotypic expression is dependent on the growth environment (Darveau *et al*, 1983; Brown and Williams, 1985; Camprubi *et al*, 1992; Allan and Poxton, 1994; Mengistu *et al*, 1994). It is possible that differences in the phenotypic expression of these structures participate in the greater resistance of *E. coli* grown in Osmolite. The phenotypic expression of capsules in response to growth in laboratory media and enteral nutrient solutions was briefly investigated using Discontinuous Percoll Gradients Centrifugation (Patrick and

Reid, 1983) and light microscopy with an India Ink Stain (Cruikshank *et al*, 1975). However, preliminary results found that components of the enteral feeding systems collapsed the Percoll gradients and washing the bacterial cells to remove these components was unacceptable because capsular material may also have been removed.

Equally, it is also possible that components from the feed are either mopping up the complement in the test medium or coating the surface of the bacteria. A coating of components present in the enteral formula could potentially cover areas of O-polysaccharide, thus preventing activation of the classical complement pathway or preventing access to the outer membrane of the bacteria in the same manner as O-polysaccharide might. It has been shown that strains of *E. coli* which have a high resistance to complement inactivation are more likely to cause bacteraemia (Jacobson *et al*, 1992). Therefore, it is possible that growth of bacteria in milk-based enteral nutrient solutions might enhance their ability to survive in the blood stream and therefore increase the risk of disease. Further study is required to determine whether growth in milk-based enteral nutrient solutions increases the serum resistance of other Gram-negative bacteria and by what mechanisms this occurs. It would be particularly useful to determine by which pathway the complement cascade was initiated. In addition, the strain of *E. coli* used in these experiments was relatively serum sensitive, it would be interesting to use a more serum resistant strain of *E. coli* to see if a similar observation could be made. These results therefore show that experiments in laboratory media do not represent an accurate model of the serum resistance of bacteria that can contaminate enteral nutrient solutions.

Another aspect of bacterial pathogenicity mediated by LPS is endotoxicity. Macrophages and monocytes produce large amounts of pro-inflammatory cytokines, reactive oxygen species and bioactive mediators in response to the lipopolysaccharide of Gram-negative bacteria (Morrison and Ryan, 1987; Wright, 1991). Measurement of cytokine and other inflammatory mediator production by these cells can be used to assess the endotoxicity of lipopolysaccharide and, for the purpose of this study, to compare lipopolysaccharides

from bacteria grown in different environments. Two of these molecules released from macrophages and monocytes are nitric oxide (a reactive oxygen species and an inflammatory mediator) and TNF $\alpha$  (a pro-inflammatory cytokine).

Nitric oxide was selected because it is a messenger molecule that has a variety of functions and is relatively cheap and easy to measure. It is the end product of the oxidation of the guanidino nitrogen of L-arginine by the enzyme nitric oxide synthase (Reiling *et al*, 1994). Three isoforms of nitric oxide synthase are recognised to be expressed by murine and human cells. Two of these are expressed constitutively, being found either surface bound in endothelial cells or in the cytoplasm of neurones from either the central or peripheral nervous systems. Both these isoforms of nitric oxide synthase produce nitric oxide in response to transient calcium fluxes, generating small amounts for a short period of time. However, the third type of nitric oxide synthase is absent in resting cells only being expressed when cells are stimulated with cytokines or lipopolysaccharide. In this case, macrophages seem to be the most important cell and large amounts of nitric oxide are produced. It is nitric oxide from this source that is active against some microbial pathogens (Nathan and Hibbs, 1991) and is also a vaso-relaxant (Stuehr and Griffith, 1992; Kelly *et al*, 1995). In minor infections this latter property may result in redistribution of blood flow, enhancing the oxygen delivery to target organs (Kelly *et al*, 1995). However, in severe infections over-production of nitric oxide can lead to dangerous hypotension, a characteristic of septic shock (Parker *et al*, 1987).

Nitric oxide is unstable in the presence of oxygen and is oxidised to either nitrite or nitrate. Its presence may be investigated in the supernatants of stimulated macrophages using a colourimetric assay which measures nitrite. Alternatively, nitrite may also be measured using gas or mass spectrometry (Green *et al*, 1982). This study used the colourimetric assay which is cheaper and easier to use. Experiments utilized hot-aqueous phenol extracts and whole bacterial cells as sources of lipopolysaccharide. Experiments

where free lipopolysaccharide from hot-phenol extractions were the subject of investigation utilized murine peritoneal macrophages because the human monocyte cell line (THP-1) does not produce nitric oxide in response to free LPS (Urayet, 1996). However, THP-1 cells do produce nitric oxide in response to whole bacteria and so were used in other experiments. However, this cell line proved difficult to culture, being very sensitive to oxygen levels in the media, freezing and the presence of antibiotics.

Only very small amounts of nitric oxide were produced by the murine peritoneal macrophage cell line in response to stimulation with free LPS ( $>8.86 \mu\text{mol per } 10^6 \text{ cells per } 24 \text{ hours}$ ). However, these were similar to the amounts found by Stuehr and Marletta (1987) who investigated nitric oxide production by murine macrophage cell lines in response to  $50 \mu\text{g ml}^{-1}$  *E. coli* and Schneemann *et al* (1993) who found that murine peritoneal macrophages produced  $5.5 \mu\text{mol per } 10^6 \text{ cells per } 24 \text{ hours}$  in response to a  $2 \mu\text{g ml}^{-1}$  concentration of *E. coli* LPS. Although, LPS from *P. aeruginosa* grown in nutrient broth induced the most nitric oxide production by murine peritoneal macrophages, no significant difference was observed between this and LPS from *P. aeruginosa* grown in the Osmolite. This may have been due to the large intra- and inter-experimental standard errors. Intra-experimental error may have been due to clumping of macrophages after counting, causing an unequal distribution of cells amongst the assay wells. The high inter-experimental error may have been as a result of differences in macrophage viability rates and also the difficulty of controlling all the variables in such experiments.

Much higher amounts of nitric oxide were produced by the THP-1 human monocyte cell line in response to whole cells than the murine macrophages in response to the extracted lipopolysaccharide. Although there were significant differences between experiments, the overall trends in the results were the same. The differences between experiments may be explained by the difficulty in maintaining the viability of THP-1 cells during experiments and problems incurred by the error associated in enumerating bacteria and adjusting

numbers to the required concentrations. Both experiments indicated that the bacteria grown in nutrient broth induced significantly less nitric oxide than when those bacteria were grown in milk-based enteral nutrient solutions. In the second experiment the amounts of nitric oxide expressed in response to bacteria grown in nutrient broth was so low that it was found not to be significantly more than the baseline production of nitric oxide by the unstimulated monocytes.

There were also differences in amounts of nitric oxide produced by THP-1 cells in response to bacteria grown in different brands of milk-based enteral nutrient solution. In both experiments, THP-1 cells produced less nitric oxide in response to bacteria grown in Osmolite than bacteria grown in either Fortisip or Pulmocare. However, there was no agreement between the experiments as to whether bacteria grown in Fortisip or bacteria grown in Pulmocare induced the most nitric oxide production in monocytes. There are a number of differences in the formulation of these milk-based enteral nutrient solutions which may have the potential to affect the expression of bacterial cell surface components. Osmolite has lesser amounts of lipids, proteins, sodium ions, iron, Vitamin E, niacin and choline compared to Pulmocare and Fortisip. However, it is difficult to speculate which (if any) of the differences in amounts of these feed components is important.

These results also highlight the need for further research in this area. Lipid A is almost entirely responsible for the endotoxic nature of LPS (Morrison and Ryan, 1987) and yet its structure is highly conserved even across different genera of bacteria (Mutharia *et al*, 1984). Therefore, it is unlikely that environmental modulation of Lipid A phenotype is responsible for differences in amounts of nitric oxide released by monocytes/macrophages in response to bacteria grown in different media. The mechanism for this remains unknown but may be related to differences in the way LPS molecules are arranged on the surface of the bacterial cells. It is also possible that other structures present on the bacterial cell surfaces are stimulating nitric oxide production by



monocytes. Urayet (1996) used polymixin B to bind to LPS, thus preventing access of THP-1 cells to LPS. This author found that although nitric oxide production by THP-1 was blocked when outer membrane preparations treated with polymixin B were used as stimulus, THP-1 cells still produced nitric oxide in response to whole cell preparations treated with polymixin B. This suggests that other bacterial components stimulate the production of pro-inflammatory mediators. A similar result was also obtained by this author when antibodies specific for core oligosaccharide were used to block access to Lipid A. It is therefore conceivable that there are other structures on the bacterial cell surface which may be subject to environmental modulation and are involved in stimulation of monocytes and macrophages to produce nitric oxide.

In addition to nitric oxide, the amount of TNF $\alpha$  was measured in the supernatants from stimulated THP-1 monocyte cells. TNF $\alpha$  is an important mediator of inflammatory and immune function regulating the growth and differentiation of many types of cells, while being selectively cytotoxic to others (Callard and Gearing, 1994). It is secreted by many types of cells including activated monocytes and macrophages, T lymphocytes, B lymphocytes and fibroblasts. It has an important role in the development of metabolic and pathological consequences of infection or invasion (Tracey, 1992). Although production in small amounts enhances the ability of the immune system to fight infection, over-production of this cytokine is associated with the symptoms of septic shock (Hinshaw, 1989). In addition it has also been implicated in the pathogenesis of cachexia, AIDS, auto-immune diseases and inflammatory diseases (Folks *et al*, 1989; Tracey, 1992).

The use of the L929 mouse fibroblast cell line bioassay to quantify TNF $\alpha$  production by macrophages was first described by Flick and Gifford (1984). The L929 cells were easy to culture, grew rapidly and were very sensitive to TNF $\alpha$ . Although other methods such as Enzyme Linked Immuno-Sorbant Assays (ELISA) have been indicated for use when measuring TNF $\alpha$  (Garde *et al*, 1995), this assay has the advantage that the biological

activity in addition to the quantity of the TNF $\alpha$  may be ascertained. Therefore, this method has been used in a number of studies (Mohler *et al*, 1993; Timmerman *et al*, 1993; Falconer *et al*, 1994; Delahooke *et al*, 1995).

Very little biologically active TNF $\alpha$  was measured in the supernatants from the monocyte cell line stimulated with any of the permutations of whole bacteria grown in different environments. This may have been because TNF $\alpha$  is produced in response to bacterial stimuli relatively quickly, TNF $\alpha$  has a short half-life and concentrations of TNF $\alpha$  were measured too long after the initial burst of TNF $\alpha$  by the monocytes. Subsequent experiments would have been conducted to establish a time versus TNF $\alpha$  production course. The limited nature of these results also made it difficult to draw conclusions about how the growth environment of the bacteria affected their ability to induce TNF $\alpha$  production by the monocyte cell lines. For example, monocytes produced significantly more TNF $\alpha$  in response to *E. coli* grown in Fortisip than *E. coli* grown in nutrient broth, Osmolite or Pulmocare. Whereas, monocytes produced significantly more TNF $\alpha$  in response to *P. aeruginosa* grown in laboratory media compared to *P. aeruginosa* grown in Fortisip, Osmolite or Pulmocare. It is possible that because the time at which the highest levels of TNF $\alpha$  were produced by the monocytes was not selected for measurement, any differences between the amounts of TNF $\alpha$  produced in response to bacteria grown in different environments were not detected. Although firm conclusions are impossible to draw from the results of nitric oxide and TNF $\alpha$  assays, results do suggest the possibility that the endotoxicity of the bacteria grown in laboratory media is different from that of bacteria grown in milk-based enteral nutrient solutions.

The third aim of this Chapter was to investigate whether sub inhibitory concentrations of oil of fennel, parabens and combinations of these preservatives altered the expression of lipopolysaccharide in laboratory media and enteral nutrient solutions, thus reducing bacterial pathogenicity. The experiments in this study showed that many of the effective combinations in enteral nutrient solution contained concentrations of methyl parabens

that were higher than the legal limit in the UK. This was particularly evident with *P. aeruginosa* which was especially resistant to oil of fennel, parabens and the combinations of those antimicrobial agents. Therefore, an alternative approach to preservation of the enteral nutrient solutions, which could reduce risk to patients, is to inhibit the harmful products produced by contaminating microorganisms and reduce the virulence of organisms. Sub-lethal concentrations of antibiotics have been shown to damage microorganisms or alter the phenotypic expression of microbial products and structures, in such a manner that the virulence may be reduced (Lorian and Gemmell, 1991; Nelson *et al*, 1993). Although, components of oil of fennel (anethole) and parabens have been shown to have an effect of toxin production in some microbial species (Robach and Pierson, 1978; Hitokoto *et al*, 1980; Draughton *et al*, 1982; Venugopal *et al*, 1984), their effect on the phenotypic expression of cell surface structure and overall microbial pathogenicity remains unknown.

Experiments were based on sub-lethal concentrations of the combination of oil of fennel and methyl parabens determined by the Checkerboard Technique in Chapter Four. However, parabens concentrations were restricted to the UK limit for use as a preservative in foods (0.1%, Preservatives in Food Regulations, 1989). Thus, these factors suggested that a sub-lethal combination of 1.0% oil of fennel and 0.1% methyl parabens should be used in experiments with *P. aeruginosa*, both in laboratory media and in milk based enteral nutrient solution. Unfortunately in these experiments, where large quantities of bacterial culture were required to enable lipopolysaccharide extraction, this combination was found to be inhibitory to the growth of *P. aeruginosa*. The reason for the greater efficacy of the preservative combination when used in large quantities is unknown. The concentration of oil of fennel was reduced to 0.5% in other experiments. This concentration of oil of fennel in the combination enabled sufficient growth of *P. aeruginosa* for lipopolysaccharide to be extracted.

SDS-PAGE gels showed that there were no changes to the O-polysaccharide portion of organisms cultured either in laboratory media or Osmolite, supplemented with sub-lethal concentrations of antimicrobial agents. This may have been due to several reasons: the maximum legal concentration of parabens was too low to exert any effect; combinations were determined using data from the Checkerboard results which was too inaccurate; or combinations of oil of fennel and parabens have no effect on the expression of LPS. However, the possibility that the pathogenicity of microorganisms could be reduced by the use of sub-lethal concentrations of these antimicrobial agents remains an intriguing possibility and one that should be researched further.

## **5.5 SUMMARY**

Growth in laboratory media such as nutrient broth does not make a good model for studies which investigate the phenotypic expression of virulence determinants and pathogenicity of bacteria growing in enteral nutrient solution. Growth in milk-based enteral nutrient solutions influences the phenotypic expression of LPS and may, as a result, change aspects of bacterial pathogenicity, such as serum resistance and endotoxicity. Addition of sub-lethal concentrations of oil of fennel, methyl parabens or combinations of these preservatives did not alter the phenotypic expression of the O-polysaccharide at the concentrations used. However, the possibility of this and other aspects of microbial pathogenicity (such as toxigenesis) may be reduced by adding these antimicrobial agents was not eliminated. The difficulties of separating bacteria for the analysis of their structural components, from complex growth media such as enteral nutrient solutions was underlined.

## CHAPTER SIX

### GENERAL DISCUSSION OF RESULTS

Enteral feeding systems contaminated with microorganisms either from exogenous sources or from the patients own commensal flora are associated with bacteraemia, diarrhoea, respiratory infections and septicaemia. However, it is only in recent times that researchers have begun to investigate the growth of microorganisms in enteral nutrient solutions and possible changes in their pathogenicity. The only two examples of this type of research are that of Rowan and Anderson (1997), and the results presented in this thesis and Hodgson *et al*, (1999).

Infant milk formula and milk-based nutritional supplements for HIV patients are similar in composition to milk-based enteral nutrient solutions. Rowan and Anderson (1998a,b) found that these types of nutritional supports may become contaminated with diarrhoeagenic *Bacillus cereus*. Furthermore, they found that maltodextrin a component of these formulas stimulated the growth and toxigenesis of *B. cereus* (Rowan and Anderson, 1997). Epidemiological data was insufficient to prove a causal link between the presence of *B. cereus* and episodes of diarrhoea in patients. It seems, however, that the maltodextrin in infant milk formula and nutritional supplements for HIV patients is probably linked to the incidence of *B. cereus* diarrhoea associated with these feeds.

In this thesis, microbial growth was similar in milk-based enteral nutrient solutions to laboratory media in most cases, indicating that it is unlikely that maltodextrin present in these types of feeds stimulates the growth of the microorganisms tested. The results indicated that growth in milk-based enteral nutrient solutions altered the expression of virulence factors of microorganisms, a finding that is similar to that of Rowan and Anderson (1997). Specifically, this work has shown that growth in these feeds alters the phenotypic expression and biological activity of lipopolysaccharide. The nature of this research, however, makes it difficult to determine if bacteria grown in these feeds have

increased pathogenicity, so we may only speculate whether the potential of these organisms to cause disease in the enterally fed is changed.

Lipopolysaccharide not only stimulates the immune system by inducing inflammatory responses and activating complement via the alternative pathway and/or the classical pathway in the presence or absence of antibody (Morrison and Kline, 1977), it also confers to bacteria resistance to host defences and antimicrobial agents. Thus, it has a central role in the pathogenesis of Gram-negative sepsis (Morrison and Ryan, 1987; Rietschel and Brade, 1992). Changes in the chain length of the O-polysaccharide portion of lipopolysaccharide molecules, the proportion of lipopolysaccharide molecules with O-polysaccharide and the stearic distribution of O-polysaccharide chains across the bacterial cell surface can alter resistance to phagocytosis, serum and antibiotics (Proctor, *et al*, 1995). Therefore, Gram-negative bacteria which have grown in milk-based enteral nutrient solutions may have an O-polysaccharide phenotype which confers an increased ability to resist these non-specific immune defences and to resist intervention with antibiotics. Data presented in this thesis demonstrated that serum sensitive bacteria increased their resistance to complement after growth in milk-based enteral nutrient solutions. However, the role of lipopolysaccharide in this increase in resistance was not confirmed. This increase in serum resistance may be as a result of phenotypic changes in other microbial structures which play a role in serum resistance, such as capsules and outer membrane proteins, or because components of the feed formed a protective layer around the bacterial cell.

An increase in bacterial resistance to serum and phagocytosis could also increase the ability of Gram-negative bacteria to survive and multiply in the bloodstream causing bacteraemia and septicaemia. In addition, there may also be an increased inflammatory response to bacteria that have grown in enteral nutrient solutions. Here, it was found that substantially more nitric oxide was released by a human monocyte cell line in response to bacteria grown in milk-based enteral nutrient solution compared to those cultured in

conventional laboratory media. If such results were observed with other inflammatory cytokines such as IL-1 and TNF $\alpha$  it might be possible that bacteria grown in enteral nutrient solutions would be more likely to cause septic shock. Thus, the growth conditions provided to bacteria by milk-based enteral nutrient solutions could increase the incidence of serious Gram-negative infections in the enterally fed patient.

Changes in the phenotypic expression of O-polysaccharide can also alter the cell surface charge of the bacteria. Makin and Beveridge (1996) found that *P. aeruginosa* which had either rough lipopolysaccharide or most of their lipopolysaccharide containing A-band O-polysaccharide were more hydrophobic. This enabled the bacteria to have increased adherence to polystyrene. In contrast, *P. aeruginosa* that produced lipopolysaccharide with mostly B-band O-polysaccharide had increased adherence to hydrophilic surfaces such as glass. These authors speculated that phenotypic change in the organisms cell surface hydrophobicity caused by environmental stimuli may influence adhesion and favour survival. Therefore, it is also possible that growth in enteral nutrient solutions may alter the expression of O-polysaccharide in such a manner that the adhesion to feeding catheters is increased. This could in turn favour the growth of bacteria up the outer surface of feed catheters, in the manner described by Payne-James *et al* (1992), causing respiratory infections.

To prevent microbial contamination and growth in enteral feeding systems, several strategies have been employed to reduce the opportunity for microorganisms to enter the feeding system. Firstly, manufacturers have promoted the use of "closed" enteral feeding systems. Secondly, organisations such as the Parenteral and Enteral Group of the British Dietetic Association have issued microbiological guidelines which cover the preparation of "mixed-by-hand" formulas, microbial criteria for open feeding systems and recommended "hang" or usage times for tube and sip feeds. Finally a minority of feeds have had potassium sorbate added to them as a preservative.



These methods of controlling microbial contamination seem to have failed. There is much anecdotal evidence to suggest that infectious complications associated with enteral feeding are commonplace. The reasons for this failure are clear. The recommended guidelines are not incorporated into the procedures of many hospitals and in any case guidelines do not seem to be followed in the clinical environment with any regularity. Despite the claims of some authors (Wagner *et al*, 1994; Dentinger *et al*, 1995, and Weenk *et al*, 1995), the incidence and amount of contamination does not seem to be significantly different in "closed" feeding systems compared to "open" feeding systems (Crocker *et al*, 1986; Chan *et al*, 1994; Donius, 1993). The principle reason for comparable levels of microorganisms in "closed" systems seems to be that, although they are much more effective at preventing exogenous contamination entering the feeding system, retrograde contamination of these feeding systems by microorganisms from the patient's own GI-tract is not prevented. Finally, potassium sorbate is ineffective as a broad-spectrum antimicrobial agent and is thus unsuitable as a preservative in enteral nutrient solutions (Anderton, 1985; Scott and Gorman, 1992).

In addition, even some of the recommendations presented in the literature appear to be ill-advised. Authors such as Wagner and co-workers (1994) and Weenk *et al* (1995) have proposed that "closed" feeding systems may be safely used for hang times of up to 48 hours. Results presented here clearly show that growth in milk-based enteral nutrients solutions, the most common type used for tube-feeding, is very rapid at room temperature. Even small numbers of bacteria introduced into the enteral feeds were found to have increased to levels in excess of  $8.00 \log \text{ cfu ml}^{-1}$  by 24 hours and in many cases by 14 hours. It is likely that such large numbers of microorganisms pose a serious risk of infection to the enterally fed patient, especially as many of these patients are immunocompromised. Results from the studies of Chan *et al*, 1994 and others suggest the microorganisms are never eliminated entirely from "closed" enteral feeding systems and therefore the risk of microbial contamination within feeding systems is always present. Extension of hang times from 24 hours would increase the potential of

microorganisms to reach very high levels in the enteral feeding system which may increase the risk of disease, deplete the nutritional content of the enteral nutrient solution and cause blockage of feed lines and catheters. Furthermore, the results from this thesis indicate that a reduction in the hang times of "closed" enteral feeding systems from 24 hours to 6 or 8 hours may ensure that the potential for high numbers of microorganisms to accumulate is reduced. Clearly, this has cost implications both in terms of administering staff time and the amount of disposable packaging required. Such costs must be balanced against the risk and possibility that alternative strategies, which are more cost effective, may be available to reduce this risk.

The most feasible of these alternative strategies for preventing the accumulation of large concentrations of microorganisms in enteral feeding systems is the inclusion of preservatives in enteral nutrient solutions. However, there are few broad-spectrum antimicrobial agents available on the market which can be used to preserve milk-based products. Therefore, the development of novel preservatives was required and the possibility that oil of fennel, parabens and combinations of these compounds may be used as preservatives in enteral nutrient solutions was investigated in this thesis.

There are several advantages to the use of chemical preservation as a method of reducing the incidence of disease associated with contaminated enteral feeds. Firstly and most importantly, chemical preservation would not only inhibit the growth of exogenous microbial contaminants but also may prevent the contamination of feed tubes and catheters with organisms from the patient's own GI-tract. Secondly, they do not depend on clinical staff following procedures which use a lot of resources and time and are thus often abused. Finally, chemically preserved enteral feeds are suitable for all types of feeding systems, including sip feeds.

However, there are potentially some disadvantages associated with chemical preservation of enteral feeds using the combinations described in this thesis. The amount of parabens

given during enteral feeding may exceed the daily recommended amounts (Preservatives in Foods Regulations, 1989). Antibiotics and other drugs given concomitantly with enteral feeds, to treat patients, may interact with oil of fennel and parabens, used as preservatives, causing a decrease in efficacy of the treatments and preservatives. In addition, the inclusion of chemical preservatives may have a disrupting influence upon the microbial flora of the patients' GI-tract. Selective inhibition or elimination of microorganisms from the gut flora can lead to the colonization by pathogens or overgrowth of a single species from the gut micro-flora. In such cases opportunistic infections may arise such as infectious enterocolitis caused by *Clostridium difficile* and candidiasis caused by overgrowth of *C. albicans* in the GI-tract. Combinations of oil of fennel and parabens may cause disturbance to the microbial flora of the gut because microorganisms showed considerable variation in their sensitivity to these combinations. Although there is no evidence to suggest that parabens (Davidson, 1993) or oil of fennel affect the gastro-intestinal flora in a detrimental way, this possibility should be investigated by studying the effect of these antimicrobial agents used singly and in combination, on mixed cultures of microorganisms.

The results here show that combinations of oil of fennel and parabens had synergistic antimicrobial activity against some of the most important strains of contaminating microorganisms in enteral feeds. Thus, it was the combinations rather than oil of fennel or parabens used as single agents, which were most suitable for inclusion as preservatives in enteral nutrient solutions. Oil of fennel and methyl parabens was the most thoroughly investigated combination in this study. However, for the purposes of preserving enteral nutrient solutions, combinations of oil of fennel with other parabens of increased alkyl chain length may be more effective. Indeed, the concentrations of methyl parabens required to inhibit the most resistant organisms tested (*Ent. cloacae* and *P. aeruginosa*) were in excess of the legal amount permitted in foods under the Preservative in Foods Regulations (1989). These regulations prohibit the use of any parabens at concentrations over 0.1% in foods sold in the UK. However, studies conducted in laboratory media

indicated that ethyl and propyl parabens in combination with oil of fennel had greater antimicrobial activity than combinations with methyl parabens (Hodgson *et al*, 1998). The use of either ethyl or propyl parabens may enable a synergistic combination preservative to be developed which utilizes a legal concentration of parabens and inhibits the growth of microbial contaminants in enteral nutrient solutions.

The potential of these antimicrobial agents to inhibit the production and activity of a variety of microbial products as well as growth was important because microbial populations which are not growing will continue to produce cell surface structures and exotoxins which can over-stimulate the immune system and disrupt the function of targeted host cells. This requirement is made more important if the pathogenicity of the microorganisms has been enhanced during growth in milk-based enteral nutrient solutions as discussed earlier. Combinations did not alter the expression of lipopolysaccharide. This may have been because the concentrations of oil of fennel and methyl parabens were too low or that oil of fennel and parabens do not in fact affect the microbial synthesis or assembly of lipopolysaccharide.

This thesis has investigated the growth, inhibition and pathogenicity of microorganisms which contaminate enteral nutrient solutions. However, it has also highlighted the need for research which will establish how enteral feeds affect the ecology and pathogenicity of the normal microbial flora of the GI-tract. Although similar numbers of all microbial strains were measured after 24 hours of growth in milk-based enteral nutrient solutions, it may be important to examine the growth of mixed cultures in these feeds. This would produce a more realistic model of the interactions between enteral nutrient solutions and microorganisms, regardless of whether those microorganisms are in the gut or are contaminants in the enteral feeding system. Another development of this study would be to investigate microbial growth and pathogenicity of microorganisms in anaerobic conditions, as found in the lower intestine.

This research has shown that growth in milk-based enteral nutrient solutions can affect the cell surface structures of microorganisms *in vitro*. However, the effects of enteral nutrition on the cell surface structures of gut microorganisms is difficult to investigate because of the huge number of variables which are present in an *in vivo* situation. Although enteral feeding may have a direct effect on the microorganisms of the gut it is at the moment almost impossible to determine what this might be. However, if the effect is to increase the pathogenicity of microorganisms in the gut then this could, in itself, cause infectious complications in the enterally fed patient. Equally it is possible that enteral feeding could cause an overall decrease in the pathogenicity of the bacterial flora of gut, thus decreasing the risk associated with translocation of the gut flora. Finally, the beneficial effects of enteral feeding systems on the status of immune function in the GI-tract may counteract any increase that the enteral nutrient solution may have on the pathogenicity of the microbial flora.

The most important priority for future research is to continue research into the effect of milk-based and, if possible, fruit-based enteral nutrient solutions on the pathogenicity of microorganisms. This could be achieved by the development of animal models and measurement of specific antibodies to virulence determinants, in enterally fed patients with diseases contracted from consuming contaminated enteral feeds. Furthermore, epidemiological studies which investigate the link between contamination of enteral feeds and disease should be undertaken. These should use DNA techniques such as PCR to confirm whether the strains of microorganisms contaminating the enteral nutrient solutions is the same strain causing disease in the patient. Of particular interest for further study is cytokine release by human macrophages and monocytes in response to bacteria grown in enteral feeds and measurement of bacterial resistance to phagocytosis and serum. Investigation should also be made of phenotypic expression of other structures and products involved in microbial pathogenicity in the presence of enteral nutrient solutions. However, for an accurate representation of the pathogenicity of gut microorganisms in the presence of enteral nutrient solutions to be determined, an *in-vitro*

model of the conditions in gut must be developed. A first step may be to add bile salts to assays. However, if research in this area is to go further a better understanding of the microbial ecology and immune system of the gut is required and a number of severe technical difficulties must be overcome.

Another area of important research is the further development of the novel preservative system presented in this thesis. Experiments should measure the activity of combinations of oil of fennel with ethyl, propyl and even butyl parabens in enteral nutrient solutions. Understanding of the mechanism of the synergism between these two antimicrobial agents may also help the development of even more effective preservative combinations which could have a wide range of pharmaceutical and non-pharmaceutical uses. Further investigation into the effect of combinations of oil of fennel and parabens on other microbial virulence factors such as outer membrane proteins, capsules and toxin production should also be attempted and may be more fruitful than the study of lipopolysaccharide.

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